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<p>(21) International Application Number: <b>PCT/US99/22384</b> (22) International Filing Date: 24 September 1999 (24.09.99) (30) Priority Data: 09/161,113 25 September 1998 (25.09.98) US 09/255,368 22 February 1999 (22.02.99) US (71) Applicant (for all designated States except US): SYNAPTIC PHARMACEUTICAL CORPORATION [US/US]; 215 Col- lege Road, Paramus, NJ 07652 (US). (72) Inventors: GERALD, Christophe, P.G.; 204B Union Street, Ridgewood, NJ 07450 (US). JONES, Kenneth, A.; 136 East Main Street, Bergenfield, NJ 07621 (US). BONINI, James, A.; 80 Manito Avenue, Oakland, NJ 07436 (US). BOROWSKY, Beth; 218 Park Street, Montclair, NJ 07042 (US). (74) Agent: WHITE, John, P.; Cooper &amp; Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: DNA ENCODING MAMMALIAN NEUROPEPTIDE FF (NPFF) RECEPTORS AND USES THEREOF</p> <p>(57) Abstract</p> <p>This invention provides isolated nucleic acids encoding mammalian NPFF receptors, purified mammalian NPFF receptors, vectors comprising nucleic acid encoding mammalian NPFF receptors, cells comprising such vectors, antibodies directed to mammalian NPFF receptors, nucleic acid probes useful for detecting nucleic acid encoding mammalian NPFF receptors, antisense oligonucleotides complementary to unique sequences of nucleic acid encoding mammalian NPFF receptors, transgenic, nonhuman animals which express DNA encoding normal or mutant mammalian NPFF receptors, methods of isolating mammalian NPFF receptors, methods of treating an abnormality that is linked to the activity of the mammalian NPFF receptors, as well as methods of determining binding of compounds to mammalian NPFF receptors, methods of identifying agonists and antagonists of NPFF receptors, and agonists and antagonists so identified.</p> <p>Applicants: Beth E. Borowsky, et al. U.S. Serial No.: 09/980,145 Filing Date: November 28, 2001 <b>Exhibit 5</b></p>		

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DNA ENCODING MAMMALIAN NEUROPEPTIDE FF (NPFF) RECEPTORS AND  
USES THEREOF

BACKGROUND OF THE INVENTION

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This application is a continuation-in-part of U.S. Serial  
No. 09/255,368, filed February 22, 1999, which is a  
continuation-in-part of U.S. Serial No. 09/161,113, filed  
September 25, 1998, the contents of both of which are hereby  
10 incorporated by reference into the subject application.

Throughout this application, various publications are  
referenced in parentheses by author and year. Full  
citations for these references may be found at the end of  
15 the specification immediately preceding the sequence  
listings and the claims. The disclosure of these  
publications in their entireties are hereby incorporated by  
reference into this application to describe more fully the  
art to which this invention pertains.

20

Neuroregulators comprise a diverse group of natural products  
that subserve or modulate communication in the nervous  
system. They include, but are not limited to,  
neuropeptides, amino acids, biogenic amines, lipids and  
25 lipid metabolites, and other metabolic byproducts. Many of  
these neuroregulator substances interact with specific cell  
surface receptors which transduce signals from the outside  
to the inside of the cell. G-protein coupled receptors  
(GPCRs) represent a major class of cell surface receptors  
30 with which many neurotransmitters interact to mediate their  
effects. GPCRs are predicted to have seven  
membrane-spanning domains and are coupled to their effectors  
via G-proteins linking receptor activation with  
intracellular biochemical sequelae such as stimulation of  
35 adenylyl cyclase.

Neuropeptide FF (NPFF) is an octapeptide isolated from  
bovine brain in 1985 by Yang and coworkers (1) using

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antibodies to the molluscan neuropeptide FMRFamide (FMRFa). FMRFamide-like immunoreactivity was observed in rat brain, spinal cord, and pituitary, suggesting the existence of mammalian homologs of the FMRFa family of invertebrate peptides. The isolation of NPFF, named for its N- and C-terminal phenylalanines (also called F8Famide) and a second mammalian peptide, NPAF (also called A18Famide), confirmed the existence of mammalian family of peptides sharing C-terminal sequence homology with FMRFa (1). Molecular cloning has revealed that NPFF and NPAF are encoded by the same gene and cleaved from a common precursor protein (2). Studies of the localization, radioligand binding, and function of NPFF-like peptides (see below) indicate they are neuromodulatory peptides whose effects are likely to be mediated by G protein-coupled receptors (for review, see 3).

NPFF, also called "morphine modulating peptide", is an endogenous modulator of opioid systems with effects on morphine analgesia, tolerance, and withdrawal (for review see 3,4). NPFF appears to represent an endogenous "anti-opioid" system in the CNS acting at specific, high-affinity receptors distinct from opiate receptors (5,6). Endogenous NPFF has been suggested to play a role in morphine tolerance: agonists of NPFF precipitate "morphine abstinence syndrome" (i.e. symptoms of morphine withdrawal) in morphine-dependent animals (7,8), while antagonists and anti-NPFF IgG restore morphine sensitivity and ameliorate symptoms of withdrawal (9-12). NPFF antagonists potentially could be useful as therapeutic agents to prevent the development of morphine tolerance, and to treat opiate addiction. NPFF has also been suggested to participate in the regulation of pain threshold, showing both "anti-opiate" effects and analgesic effects depending on test system and route of administration (for review, see 4). As an anti-opiate, NPFF has been shown to inhibit morphine- and stress-induced analgesia (1, 13, 14, 15), whereas anti-NPFF IgG (which blocks the biological activity of NPFF)



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potentiates these two phenomena (16, 17). An NPFF antagonist may be clinically useful in potentiating the analgesic effects of morphine, allowing use of lower doses without the development of tolerance. NPFF agonists may also exhibit analgesic activity in some model systems (14, 18, 19). The analgesia elicited by NPFF is typically sensitive to naloxone, indicating that it is mediated by release of endogenous opioid peptides (19, 20). The interaction of NPFF and opioid systems in regulating pain pathways is thus complex and may involve multiple mechanisms and sites of action. NPFF has additional biological activities in accord with its pattern of expression in the nervous system.

NPFF peptide localization in rat CNS was examined using specific antibodies ((21-23); see also (3)). The highest levels of NPFF are found in spinal cord and posterior pituitary; pituitary NPFF is believed to originate in the hypothalamus. In the brain, immunoreactive cell bodies are found in two major cell groups: medial hypothalamus (between dorsomedial and ventromedial) and nucleus of the solitary tract. Immunoreactive fibers are observed in lateral septal nucleus, amygdala, hypothalamus, nucleus of solitary tract, ventral medulla, trigeminal complex, and dorsal horn of spinal cord. This localization pattern is consistent with a role for NPFF in sensory processing and modulation of opioid systems. In addition, its presence in the hypothalamus and other limbic structures could subserve roles in the regulation of appetitive and affective states. In the periphery, NPFF-like immunoreactivity (as well as NPFF binding) has been observed in the heart (24). In addition, injection of NPFF raises blood pressure in rats (24, 25). These observations, combined with the colocalization of NPFF with catecholaminergic neurons in the nucleus of the solitary tract (26), suggest that NPFF is involved in central and peripheral cardiovascular regulation.

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The ability of NPFF peptides to modulate the opioid system raised the possibility that NPFF interacts directly with opiate receptors. However, radioligand binding assays using a tyrosine-substituted NPFF analog [<sup>125</sup>I]Y8Fa demonstrate that NPFF acts through specific high affinity binding sites distinct from opiate receptors (27-30) that are sensitive to inhibition by guanine nucleotides (31). The latter observation indicates that NPFF receptors are likely to belong to the superfamily of G protein-coupled receptors which share common structural motifs. However, no reports of cloning NPFF receptors have appeared as yet.

To address the issue of potential degradation of the peptide radioligand, a more stable NPFF analog (called (1DMe)Y8Fa(18)) has also been radioiodinated and the binding characterized in spinal cord membranes (32). The binding was saturable and of high affinity; inhibition of binding with unlabeled NPFF analogs yielded K<sub>i</sub> values of 0.16nM and 0.29nM for (1DMe)Y8Fa and NPFF, respectively, with a B<sub>max</sub> = 15 fmol/mg protein. No inhibition by various opioid compounds (naloxone, morphine, enkephalins, dynorphins, etc.) or other peptides (NPY, SP, CGRP, for examples) was observed at a concentration of 10 μM, confirming the specificity of NPFF receptors. Interestingly, the related molluscan peptide FMRFa inhibited the binding of [<sup>125</sup>I](1DMe)Y8Fa with a K<sub>i</sub> = 30 nM. The effectiveness of FMRFamide and the C-terminal fragment NPFF(6-8) at NPFF receptors suggests an important role for the common C-terminus. Full activity is retained by NPFF (3-8); it has been suggested that although the C-terminus is important for receptor recognition, the N-terminus is necessary for formation of a high-affinity conformation (33).

Allard et al. (29) examined the distribution of NPFF binding sites in rat brain and spinal cord using [<sup>125</sup>I]Y8Fa ([<sup>125</sup>I]YLFQPQRFamide). The highest densities were observed in the external layers of dorsal horn of spinal cord, several brainstem nuclei, the suprachiasmatic nucleus,

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restricted nuclei of the thalamus, and the presubiculum of the hippocampus. Lower densities were seen in central gray, reticular formation, ventral tegmental area, lateral and anterior hypothalamus, medial preoptic area, lateral septum, the head of caudate-putamen and cingulate cortex. No binding was observed in cortex, nucleus accumbens, hippocampus (except in presubiculum) or cerebellum. The localization of NPFF binding sites is in good agreement with the location of the peptide itself, consistent with the binding sites mediating the biological actions of NPFF in these tissues (29, 34, 35). Less is known about the signal transduction pathways activated by NPFF receptors; NPFF was shown to activate adenylyl cyclase in mouse olfactory bulb membranes (36) but no other reports of functional coupling via G proteins have appeared.

Until now, no direct evidence for NPFF receptor subtypes has been reported in mammals. Recent physiological data suggest complex (biphasic) effects on nociception and antioptive activity of NPFF (for review, see (3, 4)) that could possibly signal the presence of multiple subtypes. Short term ICV injection of NPFF causes a hyperesthetic effect followed by long lasting analgesic effect. Intrathecal NPFF and FMRFa both produce long-lasting analgesia, but subeffective doses caused different modulatory effects on morphine-induced analgesia (F8Fa potentiated, FMRFa decreased). The analgesic effects of NPFF are sensitive to naloxone, suggesting that NPFF receptors may have distinct presynaptic (possibly associated with increase release of opioids) and postsynaptic (anti-opiate) effects mediated by multiple receptors. Little is known of the biological effects of A18Famide, which shares its C-terminal 4 amino acids with NPFF, but the existence of a family of related peptides often is predictive of multiple receptor subtypes.

No nonpeptide agonists or antagonists of NPFF are available, but several useful peptidic analogs have been developed that

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exhibit increased agonist stability or antagonist activity. For example, desamino Y8Fa (daY8Fa) can antagonize the behavioral effects of NPFF and restore morphine-sensitivity (tail-flick analgesia) to morphine-tolerant rats at lower doses, although at higher doses it can act as NPFF agonist (10) (see also (3)). (1DMe)Y8Fa, in which L-Phe<sup>1</sup> is replaced by D-Tyr and the second peptidic bond is N-methylated, has been shown to inhibit morphine-induced analgesia (18), and has higher affinity and stability than Y8Fa: (1DMe)Y8Fa was 90% stable after 150 min. incubation with rat spinal cord membranes compared with Y8Fa, which was fully degraded after 30 minutes. These analogs may be useful in predicting the effects of agonist or antagonist drugs that would act at NPFF receptors.

15

Despite the numerous studies linking NPFF with analgesia (for review, see (4)), only recently has NPFF been observed to play a role in animal models of chronic pain. For example, NPFF has recently been shown to be involved in inflammatory pain (37) and neuropathic pain (38). Importantly, NPFF was shown to attenuate the allodynia associated with neuropathic pain, suggesting that it may be clinically useful in treating this condition. In addition to its potential therapeutic roles in the treatment of pain and morphine tolerance ((4) and above), NPFF and related peptides have a number of other biological activities that may be therapeutically relevant. NPFF and FMRFamide have been shown to reduce deprivation- and morphine-induced feeding in rats (39-41), indicating that NPFF receptors may be important targets in the treatment of eating disorders. FMRFamide has also been shown to produce antipsychotic (42) and antianxiety (85) effects in rats, indicating that NPFF receptors may be valuable targets for the treatment of psychosis and anxiety. There is evidence for a role of NPFF in learning and memory. Kavaliers and Colwell (79) have shown that i.c.v. administered NPFF has a biphasic effect of spatial learning in mice: low doses improve and high doses impair learning. This suggests the possibility that

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different NPFF receptor subtypes may have opposite roles in some types of learning behavior. NPFF is known to have indirect effects on water and electrolyte balance. Arima et al. (86) have shown that NPFF will reduce increase in vasopressin release produced by salt loading or hypovolemia. Additionally, NPFF may be involved in the control of plasma aldosterone levels (87). These observations raise the possibility that agents targeting NPFF receptors may be of value in the treatment of diuresis or in the treatment of cardiovascular conditions such as hypertension and congestive heart failure. Drugs acting at NPFF receptors may be of value in the treatment of diabetes, since NPFF and A-18-Famide have been shown to produce significant inhibition of glucose- and arginine-induced insulin release in rats (88). Several investigators have reported effects of NPFF and analogs on intestinal motility in mice (89) and guinea pigs (90, 91). When administered to isolated preparations of guinea pig ileum, the actions of NPFF oppose those of opioids. Conversely, i.c.v. administration of NPFF in mice produces effects similar to those of morphine on intestinal motility. Together, these results indicate a complex modulatory role for NPFF in intestinal motility, but indicate that NPFF receptors are potential targets for drugs to treat GI motility disorders, including irritable bowel syndrome. NPFF has been shown to precipitate nicotine abstinence syndrome in a rodent model (43). These authors have raised the possibility that nicotine dependence may be attenuated by measures which inactivate NPFF. Thus, NPFF receptor antagonists may be of use for this purpose. Finally, NPFF is known to elicit two acute cardiovascular responses when administered peripherally: elevation of blood pressure and heart rate (24, 25). These actions may be mediated peripherally, centrally, or both. Thus, agents acting at NPFF receptors may be of value in the treatment of hypertension (also see above) or hypotension. The cloning of NPFF receptors will facilitate the elucidation of the roles of NPFF and related peptides in these and other important biological functions.

Described herein is the isolation and characterization of a new family of neuropeptide FF (NPFF) receptors, referred to herein as the NPFF receptors. Cloned NPFF receptors will serve as invaluable tools for drug design for pathophysiological conditions such as memory loss, affective disorders, schizophrenia, pain, hypertension, locomotor problems, circadian rhythm disorders, eating/body weight disorders, sexual/reproductive disorders, nasal congestion, diarrhea, gastrointestinal, and cardiovascular disorders.

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid encoding a mammalian NPFF receptor.

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This invention provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 1 (SEQ ID NO: 1) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

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15 This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 4 (SEQ ID NO: 3) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement. This invention also provides a

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25 nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 7 (SEQ ID NO: 5) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a

30 change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

35

This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 11 (SEQ ID NO: 7) under low stringency

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conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figures 22A-C (SEQ ID NO: 43) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

This invention also provides a purified mammalian NPFF receptor protein.

This invention further provides a vector comprising a nucleic acid encoding a mammalian NPFF receptor, particularly a vector adapted for expression of the mammalian NPFF receptor in mammalian or non-mammalian cells.

This invention provides a plasmid designated pEXJ-rNPFF1 (ATCC Accession No. 203184). This invention also provides a plasmid designated pWE15-hNPFF1 (ATCC Accession No. 203183). This invention further provides a plasmid designated pCDNA3.1-hNPFF2b (ATCC Accession No. 203255). This invention still further provides a plasmid designated pCDNA3.1-hNPFF1 (ATCC Accession No. 203605). This invention still further provides a plasmid designated pCDNA3.1-rNPFF2-f (ATCC Patent Deposit Designation No. PTA-535).

This invention additionally provides a cell comprising a



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vector which in turn comprises a nucleic acid encoding a mammalian NPFF receptor as well as a membrane preparation isolated from such a cell.

5 Moreover, this invention provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence  
10 corresponding to a sequence present within one of the two strands of the nucleic acid encoding the mammalian NPFF1 receptor and contained in plasmid pEXJ-rNPFF1 (ATCC Accession No. 203184), plasmid pWE15-hNPFF1 (ATCC Accession No. 203183), plasmid pCDNA3.1-hNPFF2b (ATCC Accession No. 203255),  
15 plasmid pCDNA3.1-hNPFF1 (ATCC Accession No. 203605) or plasmid pCDNA3.1-rNPFF2-f (ATCC Patent Deposit Designation No. PTA-535).

This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically  
20 hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1 (SEQ ID NO: 1) or (b) the reverse complement thereto.

25 This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence  
30 corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 4 (SEQ ID NO: 3) or (b) the reverse complement thereto.

35 This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic

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acid sequence shown in Figure 7 (SEQ ID NO: 5) or (b) the reverse complement thereto.

5 This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 11 (SEQ ID NO: 7) or (b) the reverse complement thereto.

10 This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figures 22A-C (SEQ ID NO: 43) or (b) the reverse complement thereto.

20 This invention still further provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding the mammalian NPFF receptor, so as to prevent translation of the RNA. This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a mammalian NPFF receptor, so as to prevent transcription thereof.

30 This invention further provides an antibody capable of binding to a mammalian NPFF receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian NPFF receptor.

35 In addition, this invention provides a pharmaceutical composition comprising (a) an amount of the oligonucleotide described above capable of passing through a cell membrane and effective to reduce expression of a mammalian NPFF receptor and (b) a pharmaceutically acceptable carrier

capable of passing through the cell membrane.

This invention also provides a transgenic, nonhuman mammal expressing DNA encoding a mammalian NPFF receptor. This invention also provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian NPFF receptor. This invention further provides a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian NPFF receptor so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian NPFF receptor and which hybridizes to mRNA encoding the mammalian NPFF receptor, thereby reducing its translation.

This invention provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor.

This invention further provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting a membrane preparation from cells transfected with DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor.

This invention provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which

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comprises separately contacting cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

This invention further provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting a membrane fraction from cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF

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receptor, under conditions permitting binding of compounds known to bind to the mammalian NPFF receptor; (c) determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

This invention also provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting a membrane preparation from cells transfected with and expressing DNA encoding a mammalian NPFF receptor with a compound known to bind to the mammalian NPFF receptor; (b) determining whether the binding of a compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (c) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

Still further, this invention provides a method of detecting expression of a mammalian NPFF receptor by detecting the presence of mRNA coding for the mammalian NPFF receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with a nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian NPFF receptor by the cell.

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5 This invention provides a method of detecting the presence of a mammalian NPFF receptor on the surface of a cell which comprises contacting the cell with an antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of the mammalian NPFF receptor on the surface of the cell.

10 This invention provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a transgenic, nonhuman mammal whose levels of mammalian NPFF receptor activity are varied by use of an inducible promoter which regulates mammalian NPFF receptor expression.

15 This invention also provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a panel of transgenic, nonhuman mammals each expressing a different amount of mammalian NPFF receptor.

20 This invention further provides a method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor comprising administering a compound to a transgenic, nonhuman mammal as described above and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian NPFF receptor, the alleviation of the abnormality identifying the compound as an antagonist. This invention also provides an antagonist identified by this method. This invention still further provides a pharmaceutical composition comprising an antagonist identified by this method and a pharmaceutically acceptable carrier.

This invention additionally provides a method of treating an abnormality in a subject wherein the abnormality is

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alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of the preceding pharmaceutical composition containing a mammalian NPFF receptor antagonist, thereby  
5 treating the abnormality.

This invention also provides a method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the  
10 activity of a mammalian NPFF receptor comprising administering a compound to a transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality  
15 identifying the compound as an agonist. This invention also provides an agonist identified by this method. This invention further provides a pharmaceutical composition comprising an agonist identified by this method and a pharmaceutically acceptable carrier. This invention  
20 provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of the preceding pharmaceutical composition containing a mammalian  
25 NPFF receptor agonist, thereby treating the abnormality..

This invention provides a method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises: (a) obtaining  
30 DNA of subjects suffering from the disorder; (b) performing a restriction digest of the DNA with a panel of restriction enzymes; (c) electrophoretically separating the resulting DNA fragments on a sizing gel; (d) contacting the resulting gel with a nucleic acid probe capable of specifically  
35 hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian NPFF receptor and labeled with a detectable marker; (e) detecting labeled bands which have hybridized to the DNA

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encoding a mammalian NPFF receptor labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; (f) preparing DNA obtained for diagnosis by steps (a)-(e); and (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

This invention provides a method of preparing a purified mammalian NPFF receptor which comprises: (a) culturing cells which express the mammalian NPFF receptor; (b) recovering the mammalian NPFF receptor from the cells; and (c) purifying the mammalian NPFF receptor so recovered.

This invention provides a method of preparing a purified mammalian NPFF receptor which comprises: (a) inserting a nucleic acid encoding the mammalian NPFF receptor into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) placing the resulting cell in suitable condition permitting the production of the mammalian NPFF receptor; (d) recovering the mammalian NPFF receptor produced by the resulting cell; and (e) isolating and/or purifying the mammalian NPFF receptor so recovered.

This invention provides a process for determining whether a chemical compound is a mammalian NPFF receptor agonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound under conditions permitting the activation of the mammalian NPFF receptor, and detecting an increase in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor agonist. This invention also provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by this process effective to increase activity of



a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

5 This invention provides a process for determining whether a chemical compound is a mammalian NPFF receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound in the presence of a known mammalian NPFF receptor agonist, under conditions permitting the activation of the  
10 mammalian NPFF receptor, and detecting a decrease in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor antagonist. This invention also provides a pharmaceutical composition which comprises an amount of a mammalian NPFF  
15 receptor antagonist determined by this process effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

20 This invention provides a process for determining whether a chemical compound specifically binds to and activates a mammalian NPFF receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor,  
25 with the chemical compound under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating  
30 that the compound activates the mammalian NPFF receptor. This invention also provides a compound determined by this process. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a NPFF receptor agonist) determined by this process effective  
35 to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a

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chemical compound specifically binds to and inhibits activation of a mammalian NPFF receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to activate the mammalian NPFF receptor, and with only the second chemical compound, under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the mammalian NPFF receptor. This invention also provides a compound determined by this process. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian NPFF receptor antagonist) determined by this effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian NPFF receptor to identify a compound which activates the mammalian NPFF receptor which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds not known to activate the mammalian NPFF receptor, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activity of the mammalian NPFF receptor is increased in the presence of the compounds; and if so (c) separately determining whether the activation of the mammalian NPFF receptor is increased by each compound included in the plurality of compounds, so as to thereby

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identify the compound which activates the mammalian NPFF receptor. This invention also provides a compound identified by this method. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian NPFF receptor agonist) identified by this method effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a method of screening a plurality of chemical compounds not known to inhibit the activation of a mammalian NPFF receptor to identify a compound which inhibits the activation of the mammalian NPFF receptor, which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds in the presence of a known mammalian NPFF receptor agonist, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activation of the mammalian NPFF receptor is reduced in the presence of the plurality of compounds, relative to the activation of the mammalian NPFF receptor in the absence of the plurality of compounds; and if so (c) separately determining the inhibition of activation of the mammalian NPFF receptor for each compound included in the plurality of compounds, so as to thereby identify the compound which inhibits the activation of the mammalian NPFF receptor. This invention also provides a compound identified by this method. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian NPFF receptor antagonist) identified by this process effective to decrease activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor agonist effective to treat the abnormality.

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5 This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor antagonist effective to treat the abnormality.

10 This invention provides a process for making a composition of matter which specifically binds to a mammalian NPFF receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor or a novel structural and functional analog or homolog thereof.

**BRIEF DESCRIPTION OF THE FIGURES****Figure 1**

5 Nucleotide sequence encoding a rat neuropeptide FF receptor (NPFF1) (SEQ ID NO: 1). In addition, partial 5' and 3' untranslated sequences are shown. In Figure 1, two start (ATG) codons (at positions 73-75 and 148-150) and the stop (TAG) codon (at positions 1369-1371) are underlined.

**Figure 2**

10 Deduced amino acid sequence (SEQ ID NO: 2) of the rat neuropeptide FF receptor (NPFF1) encoded by the nucleotide sequence shown Figures 1 (SEQ ID NO: 1).

**Figure 3**

15 Deduced amino acid sequence for rat NPFF1 (SEQ ID NO: 2). Seven solid lines designated I-VII located above portions of the sequence indicate the seven putative transmembrane (TM) spanning regions.

20

**Figure 4**

Partial coding sequence of human neuropeptide FF receptor (NPFF1) (SEQ ID NO: 3).

**Figure 5**

25 Partial deduced amino acid sequence of the human neuropeptide FF (NPFF1) receptor (SEQ ID NO: 4) encoded by the partial nucleotide sequence of Figure 3.

**Figure 6**

30 Partial amino acid alignment of rat and human NPFF1. Vertical lines represent identical residues and dots represent similar residues.

35

**Figure 7**

Nucleotide sequence of hNPFF2b (SEQ ID NO: 5). The initiating methionine and the stop codon are underlined.

**Figure 8**

Deduced amino acid sequence of human NPFF2b (hNPFF2) (SEQ ID NO: 6) encoded by the nucleotide sequence shown in Figure 7.

**Figure 9**

Deduced amino acid sequence for human hNPFF2 (SEQ ID NO: 6), with potential transmembrane domains underlined.

**Figure 10**

Amino acid alignment of rat NPFF1 and human NPFF2. Vertical lines represent identical residues and dots represent similar residues.

Figure Legends

**Figure 11**

Nucleotide sequence of a human neuropeptide FF receptor (NPFF1) (SEQ ID NO: 7). The initiating methionine (at positions 1-3) and the stop codon (at positions 1291-1293) are underlined.

**Figure 12**

Deduced amino acid sequence of the human neuropeptide FF receptor (NPFF1) (SEQ ID NO: 8).

**Figure 13**

Deduced amino acid sequence for human NPFF1 (SEQ ID NO: 8). Seven solid lines designated I-VII indicate the seven putative transmembrane (TM) spanning regions.

**Figure 14**

Amino acid alignment of the human NPFF1 and human NPFF2 receptors. Vertical lines represent identical residues and dots represent similar residues.

**Figure 15A-15C**

Electrophysiological responses to NPFF and related peptides from voltage clamped oocytes expressing NPFF1 and chimeric G-protein.

**Figures 16A-16C**

Electrophysiological responses in voltage-clamped oocytes expressing NPFF2 mRNA. **Figure 16A:** Oocyte injected with NPFF2 mRNA (from ligation PCR) generates an inward current in response to NPFF at 1  $\mu$ M. **Figure 16B:** In a different oocyte, no response is observed when challenged with a mixture of galanin, NPY, orexin A and neurokinin A, each at 1  $\mu$ M. A subsequent application of NPFF elicits a response. **Figure 16C:** Oocyte injected with NPFF2 mRNA (from BO89) generates an inward current in response to NPFF at 1  $\mu$ M. Oocytes were clamped at a holding potential of -80mV.

**Figures 17A and 17B**

Microphysiometric response of CHO cells transiently transfected with either NPFF1 (SN2) alone or NPFF1 accompanied by Gq/Gz. **Figure 17A:** Cells expressing either NPFF1 alone or NPFF1+Gq/Gz produced a dose-dependent response to NPFF with an EC50 value of 19.3 nM and 27.7 nM respectively. Mock control cells transfected with empty vector produced little if any response to NPFF even at the highest concentrations used. **Figure 17B:** Cells expressing NPFF1 alone produced a dose-dependent response to A-18-F-amide with an EC50 value of 150nM. In both Figures 17A and 17B control cells mock transfected with empty vector produced little if any response to drug even at the highest concentrations used. Responses are reported as percentage increase in the acidification rate as observed just prior to drug challenge (immediate prior basal rate).

**Figures 18A and 18B**

NPFF stimulation of Inositol phosphate release in NPFF-1 transfected Cos-7 cells. **Figure 18A:** Cos-7 cells were transiently transfected with NPFF-1 receptor cDNA. **Figure 18B:** Cos-7 cells were transiently co-transfected with cDNAs for the NPFF-1 receptor and the Gq/Gz chimera. The accumulation of total inositol phosphate release was measured by prelabelling cells with [ $^3$ H]myoinositol (2 $\mu$ Ci/ml) overnight. Cells were washed to remove unincorporated

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radioactivity and resuspended in medium containing 10mM LiCl. [<sup>3</sup>H]myoinositol labeled cells were incubated with appropriate drugs for 1hr at 37°C. The reaction was stopped by addition of 5% TCA and IPs were isolated by ion exchange chromatography (Berridge et al., 1982). Columns were washed with water and total [<sup>3</sup>H] inositol phosphates were then eluted with 1M ammonium formate/0.1 M formic acid. Radioactivity in the final fraction was measured by liquid scintillation spectroscopy. Cells were either treated with vehicle (water, control) or cholera toxin (CTX; 1 µg/ml) or pertussis toxin (PTX, 100 ng/ml) overnight. Data are from one experiment representative of at least one other.

#### Figure 19

RT-PCR was performed as described on a panel of mRNA extracted from rat tissue as indicated at the bottom of the gel. After amplification, PCR reactions were size fractionated on 10% polyacrylamide gels, and stained with SYBR Green I. Images were analyzed using a Molecular Dynamics Storm 860 workstation. The amplified band corresponding to NPFF1 (490 base pairs) is indicated (arrow). RT-PCR indicates a broad distribution of mRNA encoding NPFF1 with highest concentrations found in nervous system structures.

#### Figure 20

Autoradiograph demonstrating hybridization of radiolabeled rat NPFF1 probe to RNA extracted from rat tissue in a solution hybridization/nuclease protection assay using <sup>32</sup>P labeled riboprobe. 2µg of RNA was used in each assay. The single band (arrow) represents mRNA coding for the NPFF1 receptors extracted from the indicated tissue. Highest levels of mRNA coding for NPFF1 are found in: hypothalamus and pituitary gland. The smaller bands representing NPFF1 mRNA from the pituitary, adrenal gland, and ovary (double arrow) may indicate a splice variant present in this tissue. Integrity of RNA was assessed using hybridization to mRNA coding for GAPDH (not shown).



**Figure 21**

RT-PCR was performed as described on a panel of mRNA extracted from tissue as indicated at the bottom of the gel. After amplification, PCR reactions were size fractionated on 10% polyacrylamide gels, and stained with SYBR Green I. Images were analyzed using a Molecular Dynamics Storm 860 workstation. The amplified band corresponding to NPFF2 receptors (approximately 325 base pairs) is indicated (arrow). RT-PCR indicates a broad distribution of mRNA encoding NPFF2 receptors. The only tissue containing mRNA coding for NPFF2 receptors were HeLa cells and Jurkat cells.

**Figures 22A-22C**

Nucleotide sequence encoding a rat neuropeptide FF receptor (NPFF2). In addition, partial 5' and 3' untranslated sequences are shown. Two start (ATG) codons (at positions 26-28 and 128-130) and the stop (TAG) codon (at positions 1277-1279) are underlined.

**Figures 23A and 23B**

Deduced amino acid sequence of the rat neuropeptide FF receptor (NPFF2) encoded by the nucleotide sequence shown in Figures 22A-22C. Seven putative transmembrane spanning regions are indicated by underlining.

**Figures 24A and 24B**

Amino acid alignment of human NPFF2 and rat NPFF2. Vertical lines represent identical residues and dots represent similar residues.

**Figures 25A and 25B**

Amino acid alignment of rat NPFF1 and rat NPFF2. Vertical lines represent identical residues and dots represent similar residues.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, the following standard abbreviations are used to indicate specific nucleotide bases:

5	A =	adenine
	G =	guanine
	C =	cytosine
	T =	thymine
10	U =	uracil
	M =	adenine or cytosine
	R =	adenine or guanine
	W =	adenine, thymine, or uracil
	S =	cytosine or guanine
15	Y =	cytosine, thymine, or uracil
	K =	guanine, thymine, or uracil
	V =	adenine, cytosine, or guanine (not thymine or uracil)
	H =	adenine, cytosine, thymine, or uracil (not guanine)
20	D =	adenine, guanine, thymine, or uracil (not cytosine)
	B =	cytosine, guanine, thymine, or uracil (not adenine)
25	N =	adenine, cytosine, guanine, thymine, or uracil (or other modified base such as inosine)
	I =	inosine

Furthermore, the term "agonist" is used throughout this application to indicate any peptide or non-peptidyl compound which increases the activity of any of the polypeptides of the subject invention. The term "antagonist" is used throughout this application to indicate any peptide or non-peptidyl compound which decreases the activity of any of the polypeptides of the subject invention.

The activity of a G-protein coupled receptor such as the polypeptides disclosed herein may be measured using any of

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a variety of functional assays in which activation of the receptor in question results in an observable change in the level of some second messenger system, including, but not limited to, adenylate cyclase, calcium mobilization, arachidonic acid release, ion channel activity, inositol phospholipid hydrolysis or guanylyl cyclase. Heterologous expression systems utilizing appropriate host cells to express the nucleic acid of the subject invention are used to obtain the desired second messenger coupling. Receptor activity may also be assayed in an oocyte expression system.

It is possible that the mammalian NPFF receptor genes contain introns and furthermore, the possibility exists that additional introns could exist in coding or non-coding regions. In addition, spliced form(s) of mRNA may encode additional amino acids either upstream of the currently defined starting methionine or within the coding region. Further, the existence and use of alternative exons is possible, whereby the mRNA may encode different amino acids within the region comprising the exon. In addition, single amino acid substitutions may arise via the mechanism of RNA editing such that the amino acid sequence of the expressed protein is different than that encoded by the original gene. (Burns et al., 1996 (82); Chu et al., 1996 (83)). Such variants may exhibit pharmacologic properties differing from the polypeptide encoded by the original gene.

This invention provides splice variants of the mammalian NPFF receptors disclosed herein. This invention further provides for alternate translation initiation sites and alternately spliced or edited variants of nucleic acids encoding the mammalian NPFF receptors of this invention.

The nucleic acids of the subject invention also include nucleic acid analogs of the rat and human NPFF receptor genes, wherein the rat NPFF1 receptor gene comprises the nucleic acid sequence shown in Fig. 1 or contained in plasmid pEXJ-rNPFF1 (ATCC Accession No. 203184); the human

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NPFF1 receptor gene comprises the nucleic acid shown in Figure 4 and contained in plasmid pWE15-hNPFF1 (ATCC Accession No. 203183); the human NPFF2 receptor gene comprises the nucleic acid shown in Figure 7 and contained in plasmid pCDNA3.1-hNPFF2b (ATCC Accession No.203255); the human NPFF1 receptor gene comprises the nucleic acid shown in Figure 11 and contained in plasmid pCDNA3.1-hNPFF1 (ATCC Accession No.203605); or the rat NPFF2 receptor gene comprises the nucleic acid shown in Figures 22A-22C and contained in plasmid pCDNA3.1-rNPFF2-f (ATCC Patent Deposit Designation No. PTA-535). Nucleic acid analogs of the rat and human NPFF receptor genes differ from the rat and human NPFF receptor genes described herein in terms of the identity or location of one or more nucleic acid bases (deletion analogs containing less than all of the nucleic acid bases shown in Figs. 1, 4, 7, 11 or 22A-C or contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f respectively, substitution analogs wherein one or more nucleic acid bases shown in Figs. 1, 4, 7, 11 or 22A-C or contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f respectively, are replaced by other nucleic acid bases, and addition analogs, wherein one or more nucleic acid bases are added to a terminal or medial portion of the nucleic acid sequence) and which encode proteins which share some or all of the properties of the proteins encoded by the nucleic acid sequences shown in Figs. 1, 4, 7, 11 or 22A-C or contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f respectively. In one embodiment of the present invention, the nucleic acid analog encodes a protein which has an amino acid sequence identical to that shown in Fig. 2, 5, 8, 12 or 23A-B or encoded by the nucleic acid sequence contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f respectively. In another embodiment, the nucleic acid analog encodes a protein having an amino acid sequence which differs from the amino acid sequences shown

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in Fig. 2, 5, 8, 12 or 23A-B or encoded by the nucleic acid contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f respectively. In a further embodiment, the protein encoded by the nucleic acid analog has a function which is the same as the function of the receptor proteins having the amino acid sequence shown in Fig. 2, 5, 8, 12 or 23A-B. In another embodiment, the function of the protein encoded by the nucleic acid analog differs from the function of the receptor protein having the amino acid sequence shown in Fig. 2, 5, 8, 12 or 23A-B. In another embodiment, the variation in the nucleic acid sequence occurs within the transmembrane (TM) region of the protein. In a further embodiment, the variation in the nucleic acid sequence occurs outside of the TM region.

This invention provides the above-described isolated nucleic acid, wherein the nucleic acid is DNA. In an embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In still another embodiment, the nucleic acid is RNA. Methods for production and manipulation of nucleic acid molecules are well known in the art.

This invention further provides nucleic acid which is degenerate with respect to the DNA encoding any of the polypeptides described herein. In an embodiment, the nucleic acid comprises a nucleotide sequence which is degenerate with respect to the nucleotide sequence shown in Figures 1 (SEQ ID NO: 1), 4 (SEQ ID NO: 3), 7 (SEQ ID NO: 5), 11 (SEQ ID NO: 7) or 22A-C (SEQ ID NO: 43) or the nucleotide sequence contained in the plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f respectively, that is, a nucleotide sequence which is translated into the same amino acid sequence.

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of the

polypeptides of this invention, but which should not produce phenotypic changes. Alternately, this invention also encompasses DNAs, cDNAs, and RNAs which hybridize to the DNA, cDNA, and RNA of the subject invention. Hybridization methods are well known to those of skill in the art.

The nucleic acids of the subject invention also include nucleic acid molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors. The creation of polypeptide analogs is well known to those of skill in the art (R.F. Spurney et al. (1997); Fong, T.M. et al. (1995); Underwood, D.J. et al. (1994); Graziano, M.P. et al. (1996); Guam X.M. et al. (1995)).

The modified polypeptides of this invention may be transfected into cells either transiently or stably using methods well-known in the art, examples of which are disclosed herein. This invention also provides for binding assays using the modified polypeptides, in which the polypeptide is expressed either transiently or in stable cell lines. This invention further provides a compound identified using a modified polypeptide in a binding assay such as the binding assays described herein.

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5 The nucleic acids described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptides by a variety of recombinant techniques. The nucleic acid molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

10 This invention provides an isolated nucleic acid encoding a mammalian NPFF receptor. In one embodiment, the nucleic acid is DNA. In another embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In another embodiment, the nucleic acid is RNA.

15 In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In one embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor. In another embodiment, the mammalian NPFF2 receptor is a rat NPFF2 receptor.

20 This invention also provides an isolated nucleic acid encoding species homologs of the NPFF receptors encoded by the nucleic acid sequence shown in Figs. 1 (SEQ ID NO: 1), 4 (SEQ ID NO: 3), 7 (SEQ ID NO: 5), 11 (SEQ ID NO: 7) or 22A-C (SEQ ID NO: 43) encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f respectively. In one embodiment, the nucleic acid encodes a mammalian NPFF receptor homolog which has substantially the same amino acid sequence as does the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f. In another embodiment, the nucleic acid

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encodes a mammalian NPFF receptor homolog which has above 65% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f; preferably above 75% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f; more preferably above 85% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f; most preferably above 95% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1, or pCDNA3.1-rNPFF2-f. In another embodiment, the mammalian NPFF receptor homolog has above 70% nucleic acid identity to the NPFF receptor gene contained in plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f; preferably above 80% nucleic acid identity to the NPFF receptor gene contained in the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f; more preferably above 90% nucleic acid identity to the NPFF receptor gene contained in the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f. Examples of methods for isolating and purifying species homologs are described elsewhere (e.g., U.S. Patent No. 5,602,024, WO94/14957, WO97/26853, WO98/15570).

In separate embodiments of the present invention, the nucleic acid encodes a NPFF receptor which has an amino acid sequence identical to that encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f. In further embodiments, the NPFF receptor has a sequence substantially the same as the amino acid sequence shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figure 23A-B (SEQ ID NO: 44). In other embodiments, the NPFF receptor has an amino acid



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sequence identical to the amino acid sequence shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figure 23A-B (SEQ ID NO: 44).

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This invention provides an isolated nucleic acid encoding a modified mammalian NPFF receptor, which differs from a mammalian NPFF receptor by having an amino acid(s) deletion, replacement, or addition in the third intracellular domain.

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This invention provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 1 (SEQ ID No: 1) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

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This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 4 (SEQ ID NO: 3) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement. This invention also provides a

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nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 7 (SEQ ID NO: 5) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

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5 This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 11 (SEQ ID NO: 7) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

15 This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figures 22A-22C (SEQ ID NO: 43) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

25 In one embodiment, the mammalian NPFF receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF receptor is a rat NPFF2 receptor. For purpose of the invention hybridization under low stringency conditions means hybridization performed at 40°C in a hybridization buffer containing 25% formamide, 5X SCC, 7mM Tris, 1X Denhardt's, 25µl/ml salmon sperm DNA. Wash at 40°C in 0.1X SCC, 0.1% SDS. Changes in pH are measured through microphysiometric measurement of receptor mediated extracellular acidification rates. Because cellular metabolism is intricately involved in a broad range of cellular events (including receptor activation of multiple messenger pathways), the use of microphysiometric

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measurements of cell metabolism can in principle provide a generic assay of cellular activity arising from the activation of any receptor regardless of the specifics of the receptor's signaling pathway. General guidelines for transient receptor expression, cell preparation and microphysiometric recording are described elsewhere (Salon, J.A. and Owicki, J.A., 1996). Receptors and/or control vectors are transiently expressed in CHO-K1 cells, by liposome mediated transfection according to the manufacturers recommendations (LipofectAMINE, GibcoBRL, Gaithersburg, MD), and maintained in Ham's F-12 complete (10% serum). A total of 10 $\mu$ g of DNA is used to transfect each 75cm<sup>2</sup> flask which had been split 24 hours prior to the transfection and judged to be 70-80% confluent at the time of transfection. 24 hours post transfection, the cells are harvested and 3 x 10<sup>5</sup> cells seeded into microphysiometer capsules. Cells are allowed to attach to the capsule membrane for an additional 24 hours; during the last 16 hours, the cells are switched to serum-free F-12 complete to minimize ill-defined metabolic stimulation caused by assorted serum factors. On the day of the experiment the cell capsules are transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum (Molecular Devices Corporation, Sunnyvale, CA) containing 0.1% fatty acid free BSA), during which a baseline measurement of basal metabolic activity is established. A standard recording protocol specifies a 100 $\mu$ l/min flow rate, with a 2 min total pump cycle which includes a 30 sec flow interruption during which the acidification rate measurement is taken. Ligand challenges involve a 1 min 20 sec exposure to the sample just prior to the first post challenge rate measurement being taken, followed by two additional pump cycles for a total of 5 min 20 sec sample exposure. Typically, drugs in a primary screen are presented to the cells at 10 $\mu$ M final concentration. Ligand samples are then washed out and the acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate

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observed just prior to challenge. Endogenous NPFF peptides include rat NPFF (FLFQPQRF-NH2) (SEQ ID NO: 45) and rat A18Fa (AGEGLSSPFWSLAAPQRF-NH2) (SEQ ID NO: 46).

5 This invention provides a purified mammalian NPFF receptor protein. In one embodiment, the purified mammalian NPFF receptor protein is a human NPFF1 receptor protein. In another embodiment, the purified mammalian NPFF receptor protein is a rat NPFF1 receptor protein. In a further  
10 embodiment, the purified mammalian NPFF receptor protein is a human NPFF2 receptor protein. In a further embodiment, the purified mammalian NPFF receptor protein is a rat NPFF2 receptor protein.

15 This invention provides a vector comprising nucleic acid encoding a mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor protein is a NPFF1 receptor protein. In another embodiment of the present invention the mammalian NPFF receptor protein is a NPFF2 receptor protein. In one  
20 embodiment, the mammalian NPFF receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a human NPFF2 receptor. In a further embodiment, the purified mammalian  
25 NPFF receptor protein is a rat NPFF2 receptor.

In an embodiment, the vector is adapted for expression in a cell which comprises the regulatory elements necessary for expression of the nucleic acid in the cell operatively  
30 linked to the nucleic acid encoding the mammalian NPFF receptor as to permit expression thereof. In separate embodiments, the cell is a bacterial cell, an amphibian cell, a yeast cell, an insect cell or a mammalian cell. In another embodiment, the vector is a baculovirus. In one  
35 embodiment, the vector is a plasmid.

This invention provides a plasmid designated pEXJ-rNPFF1 (ATCC Accession No. 203184). This plasmid comprises the

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regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the mammalian NPFF1 receptor so as to permit expression thereof. This invention also provides a plasmid designated pWE15-hNPFF1 (ATCC Accession No. 203183). This invention further provides a plasmid designated pCDNA3.1-hNPFF2b (ATCC Accession No. 203255). This invention additionally provides a plasmid designated pCDNA3.1-hNPFF1 (ATCC Accession No. 203605). This invention additionally provides a plasmid designated pCDNA3.1-rNPFF2-f (ATCC Patent Deposit Designation No. PTA-535).

These plasmids (pEXJ-rNPFF1 and pWE15-hNPFF1) were deposited on September 9, 1998, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and were accorded ATCC Accession Nos. 203184 and 203183, respectively. Plasmid pCDNA3.1-hNPFF2b was deposited on September 22, 1998, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 203255. Plasmid pCDNA3.1-hNPFF1 was deposited on January 21, 1999, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 203605. Plasmid pCDNA3.1-rNPFF2-f was deposited on August 17, 1999, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent

Procedure and was accorded ATCC Patent Deposit Designation No. PTA-535.

5 This invention further provides for any vector or plasmid which comprises modified untranslated sequences, which are beneficial for expression in desired host cells or for use in binding or functional assays. For example, a vector or plasmid with untranslated sequences of varying lengths may express differing amounts of the polypeptide depending upon  
10 the host cell used. In an embodiment, the vector or plasmid comprises the coding sequence of the polypeptide and the regulatory elements necessary for expression in the host cell.

15 This invention provides a cell comprising a vector comprising a nucleic acid encoding the mammalian NPFF receptor. In an embodiment, the cell is a non-mammalian cell. In a further embodiment, the non-mammalian cell is a *Xenopus* oocyte cell or a *Xenopus* melanophore cell. In  
20 another embodiment, the cell is a mammalian cell. In a further embodiment, the mammalian cell is a COS-7 cell, a 293 human embryonic kidney cell (HEK-293 cell), a NIH-3T3 cell, a LM(tk-) cell, a mouse Y1 cell, or a CHO cell.

25 This invention provides an insect cell comprising a vector adapted for expression in an insect cell which comprises a nucleic acid encoding a mammalian NPFF receptor. In another embodiment, the insect cell is an Sf9 cell, an Sf21 cell or a *Trichoplusia ni* 5B1-4 (HighFive) cell.

30 This invention provides a membrane preparation isolated from any one of the cells described above.

35 This invention provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within one of the two strands of the

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nucleic acid encoding the mammalian NPFF receptor and are contained in plasmid pEXJ-rNPFF1, plasmid pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1 (SEQ ID NO: 1) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 4 (SEQ ID NO: 3) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 7 (SEQ ID NO: 5) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 11 (SEQ ID NO: 7) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figures 22A-22C (SEQ ID NO: 43) or (b) the reverse complement thereto. In one embodiment, the nucleic acid is DNA. In another embodiment, the nucleic acid is RNA.

As used herein, the phrase "specifically hybridizing" means

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the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

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Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes the polypeptides of this invention into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

10

RNA probes may be generated by inserting the DNA molecule which encodes the polypeptides of this invention downstream of a bacteriophage promoter such as T3, T7, or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

20

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This invention provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding a mammalian NPFF receptor, so as to prevent translation of the RNA. This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a mammalian NPFF receptor, so as to prevent translation of the genomic DNA. In one embodiment, the oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.

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35

This invention provides an antibody capable of binding to a mammalian NPFF receptor encoded by a nucleic acid encoding



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a mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF receptor is a rat NPFF2 receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian NPFF receptor. In one embodiment, the antibody is a monoclonal antibody or antisera.

This invention provides a pharmaceutical composition comprising (a) an amount of the oligonucleotide capable of passing through a cell membrane and effective to reduce expression of a mammalian NPFF receptor and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. In an embodiment, the oligonucleotide is coupled to a substance which inactivates mRNA. In a further embodiment, the substance which inactivates mRNA is a ribozyme. In another embodiment, the pharmaceutically acceptable carrier comprises a structure which binds to a mammalian NPFF receptor on a cell capable of being taken up by the cells after binding to the structure. In a further embodiment, the pharmaceutically acceptable carrier is capable of binding to a mammalian NPFF receptor which is specific for a selected cell type.

This invention provides a pharmaceutical composition which comprises an amount of an antibody effective to block binding of a ligand to a human NPFF receptor and a pharmaceutically acceptable carrier.

As used herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

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This invention provides a transgenic, nonhuman mammal expressing DNA encoding a mammalian NPFF receptor. This invention also provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian NPFF receptor. This invention further provides a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian NPFF receptor so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian NPFF receptor and which hybridizes to mRNA encoding the mammalian NPFF receptor, thereby reducing its translation. In an embodiment, the DNA encoding the mammalian NPFF receptor additionally comprises an inducible promoter. In another embodiment, the DNA encoding the mammalian NPFF receptor additionally comprises tissue specific regulatory elements. In a further embodiment, the transgenic, nonhuman mammal is a mouse.

Animal model systems which elucidate the physiological and behavioral roles of the polypeptides of this invention are produced by creating transgenic animals in which the activity of the polypeptide is either increased or decreased, or the amino acid sequence of the expressed polypeptide is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding the polypeptide, by microinjection, electroporation, retroviral transfection or other means well known to those in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these polypeptide sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native polypeptides but does express, for example, an inserted

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mutant polypeptide, which has replaced the native polypeptide in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added polypeptides, resulting in overexpression of the polypeptides.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a polypeptide of this invention is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively, or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse ( a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant ), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

This invention provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under

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conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor. This invention also provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting a membrane fraction from a cell extract of cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor. In one embodiment, the NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In one embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a rat NPFF2 receptor. In another embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as the NPFF receptor encoded by plasmid pEXJ-rNPFF1, plasmid pWE15-hNPFF1, plasmid pCDNA3.1-hNPFF2b, plasmid pCDNA3.1-hNPFF1, or plasmid pCDNA3.1-rNPFF2-f. In a further embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figures 23A-23B (SEQ ID NO: 44). In another embodiment, the mammalian NPFF receptor has the amino acid sequence shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4) Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figure 23A-23B (SEQ ID NO: 44). In one embodiment, the compound is not previously known to bind to a mammalian NPFF receptor. This invention further provides a compound identified by the above-described processes.

In one embodiment of the above-described processes, the cell is an insect cell. In another embodiment, the cell is a

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mammalian cell. In a further embodiment, the cell is nonneuronal in origin. In a further embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell. In an embodiment, the compound is a compound not previously known to bind to a mammalian NPFF receptor. This invention also provides a compound identified by the above-described process.

This invention provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

This invention also provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting a membrane preparation from cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the

presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

5 In an embodiment of the present invention, the second chemical compound is NPFF or a homolog or analog of NPFF.

10 In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the NPFF2 receptor is a human NPFF2 receptor. In a further embodiment, the NPFF2 receptor is a rat NPFF2 receptor. In another embodiment, 15 the mammalian NPFF receptor has substantially the same amino acid sequence as the NPFF receptor encoded by plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f. In a further embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figures 23A-B (SEQ ID NO: 44). In another embodiment, the mammalian NPFF receptor has the amino acid sequence shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figures 23A-B (SEQ ID NO: 44). 20 25

30 In one embodiment, the cell is an insect cell. In another embodiment, the cell is a mammalian cell. In a further embodiment, the cell is nonneuronal in origin. In another embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell. In one embodiment, the compound is not previously known to bind to a mammalian NPFF receptor. 35

This invention provides a compound identified by the above-described processes.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting  
5 cells transfected with and expressing DNA encoding the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF  
10 receptor, under conditions permitting binding of compounds known to bind the mammalian NPFF receptor; (c) determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the  
15 binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF  
20 receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting  
25 a membrane preparation from cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor under conditions permitting binding of compounds known to bind to the mammalian NPFF receptor;  
30 (b) determining whether the binding of a compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (c) separately  
35 determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to

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the mammalian NPFF receptor.

5 This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting a membrane preparation from cells transfected with and expressing the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor; (b) 10 contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor, under conditions permitting binding of compounds known to bind the mammalian NPFF receptor; (c) 15 determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian NPFF receptor of 20 compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

In one embodiment of the above-described methods, the 25 mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a 30 further embodiment the NPFF2 receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a rat NPFF2 receptor. In another embodiment, the cell is a mammalian cell. In a further embodiment, the mammalian cell is non-neuronal in origin. In another 35 embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell, or an NIH-3T3 cell.



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This invention also provides a method of detecting expression of a mammalian NPFF receptor by detecting the presence of mRNA coding for the mammalian NPFF receptor which comprises obtaining total mRNA from the cell and  
5 contacting the mRNA so obtained from a nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian NPFF receptor by the cell.

10 This invention further provides a method of detecting the presence of a mammalian NPFF receptor on the surface of a cell which comprises contacting the cell with an antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to  
15 the cell, and thereby detecting the presence of the mammalian NPFF receptor on the surface of the cell.

This invention provides a method of determining the physiological effects of varying levels of activity of  
20 mammalian NPFF receptors which comprises producing a transgenic, nonhuman mammal whose levels of mammalian NPFF receptor activity are varied by use of an inducible promoter which regulates mammalian NPFF receptor expression.

25 This invention also provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a panel of transgenic, nonhuman mammals each expressing a different amount of mammalian NPFF receptor.

30 This invention provides a method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor comprising administering a compound  
35 to a transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian NPFF receptor,

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the alleviation of the abnormality identifying the compound as an antagonist. This invention also provides an antagonist identified by the above-described method. This invention further provides a pharmaceutical composition comprising an antagonist identified by the above-described method and a pharmaceutically acceptable carrier. This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of this pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor comprising administering a compound to transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as an agonist. This invention also provides an agonist identified by the above-described method. This invention further provides a pharmaceutical composition comprising an agonist identified by the above-described method and a pharmaceutically acceptable carrier. This invention further provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of this pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises: (a) obtaining DNA of subjects suffering from the disorder; (b) performing a restriction digest of the DNA with a panel of restriction

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enzymes; (c) electrophoretically separating the resulting DNA fragments on a sizing gel; (d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian NPFF receptor and labeled with a detectable marker; (e) detecting labeled bands which have hybridized to the DNA encoding a mammalian NPFF receptor labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; (f) preparing DNA obtained for diagnosis by steps (a)-(e); and (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same. In one embodiment, a disorder associated with the activity of a specific mammalian allele is diagnosed.

This invention provides a method of preparing the purified mammalian NPFF receptor which comprises: (a) inducing cells to express the mammalian NPFF receptor; (b) recovering the mammalian NPFF receptor from the induced cells; and (c) purifying the mammalian NPFF receptor so recovered.

This invention provides a method of preparing the purified mammalian NPFF receptor which comprises: (a) inserting nucleic acid encoding the mammalian NPFF receptor in a suitable vector; (b) introducing the resulting vector in a suitable host cell; (c) placing the resulting cell in suitable condition permitting the production of the isolated mammalian NPFF receptor; (d) recovering the mammalian NPFF receptor produced by the resulting cell; and (e) purifying the mammalian NPFF receptor so recovered.

This invention provides a process for determining whether a chemical compound is a mammalian NPFF receptor agonist which comprises contacting cells transfected with and expressing

DNA encoding the mammalian NPFF receptor with the compound under conditions permitting the activation of the mammalian NPFF receptor, and detecting an increase in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor agonist. This invention also provides a process for determining whether a chemical compound is a mammalian NPFF1 receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound in the presence of a known mammalian NPFF receptor agonist, under conditions permitting the activation of the mammalian NPFF receptor, and detecting a decrease in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor antagonist. In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In one embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a rat NPFF2 receptor.

This invention further provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by the above-described process effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor agonist is not previously known.

This invention provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by the above-described process effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor antagonist is not previously known.

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This invention provides a process for determining whether a chemical compound specifically binds to and activates a mammalian NPFF receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the chemical compound under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the mammalian NPFF receptor. In one embodiment, the second messenger response comprises chloride channel activation and the change in second messenger is an increase in the level of inward chloride current.

This invention also provides a process for determining whether a chemical compound specifically binds to and inhibits activation of a mammalian NPFF receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to activate the mammalian NPFF receptor, and with only the second chemical compound, under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the mammalian NPFF receptor. In one embodiment, the second messenger response comprises chloride channel activation and the change in second messenger response is a smaller

increase in the level of inward chloride current in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound. This invention also provides the above-described processes performed with membrane preparations from cells producing a second messenger response and transfected with and expressing the mammalian NPFF receptor.

In one embodiment of the above-described processes, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a rat NPFF2 receptor. In another embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, pCDNA3.1-hNPFF1 or pCDNA3.1-rNPFF2-f. In a further embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figures 23A-B (SEQ ID NO: 44). In another embodiment, the mammalian NPFF receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 2 (SEQ ID NO: 2), Figure 5 (SEQ ID NO: 4), Figure 8 (SEQ ID NO: 6), Figure 12 (SEQ ID NO: 8) or Figures 23A-B (SEQ ID NO: 44). In an embodiment, the cell is an insect cell. In a further embodiment, the cell is a mammalian cell. In a still further embodiment, the mammalian cell is nonneuronal in origin. In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell. In an embodiment, the compound is not previously known to bind to a mammalian NPFF receptor. This invention also provides a

compound determined by the above-described processes.

5 This invention also provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by the above-described processes effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor agonist is not previously known.

10 This invention further provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by the above-described processes effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor antagonist is not previously known.

20 This invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian NPFF receptor to identify a compound which activates the mammalian NPFF receptor which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds not known to activate the mammalian NPFF receptor, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activity of the mammalian NPFF receptor is increased in the presence of the compounds; and if so (c) separately determining whether the activation of the mammalian NPFF receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound which activates the mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor is a human NPFF receptor. In a further embodiment the human NPFF receptor is a human NPFF1 receptor or a human NPFF2 receptor.

This invention provides a method of screening a plurality of

chemical compounds not known to inhibit the activation of a mammalian NPFF receptor to identify a compound which inhibits the activation of the mammalian NPFF receptor, which comprises: (a) contacting cells transfected with and  
5 expressing the mammalian NPFF receptor with the plurality of compounds in the presence of a known mammalian NPFF receptor agonist, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activation of the mammalian NPFF receptor is reduced in the  
10 presence of the plurality of compounds, relative to the activation of the mammalian NPFF receptor in the absence of the plurality of compounds; and if so (c) separately determining the inhibition of activation of the mammalian NPFF receptor for each compound included in the plurality of  
15 compounds, so as to thereby identify the compound which inhibits the activation of the mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the NPFF1 receptor is a human NPFF1 receptor. In another  
20 embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the NPFF2 receptor is a human NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a rat NPFF2 receptor.

25 In one embodiment of the above-described methods, the cell is a mammalian cell. In another embodiment, the mammalian cell is non-neuronal in origin. In a further embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic  
30 kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

This invention provides a pharmaceutical composition comprising a compound identified by the above-described methods effective to increase mammalian NPFF receptor  
35 activity and a pharmaceutically acceptable carrier.

This invention also provides a pharmaceutical composition comprising a compound identified by the above-described



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methods effective to decrease mammalian NPFF receptor activity and a pharmaceutically acceptable carrier.

5 This invention further provides a method of measuring polypeptide activation in an oocyte expression system such as a *Xenopus* oocyte expression system or melanophore. In an embodiment, polypeptide activation is determined by measurement of ion channel activity. In another embodiment, polypeptide activation is measured by aequorin luminescence.

10 Expression of genes in *Xenopus* oocytes is well known in the art (Coleman, A., 1984; Masu, Y., et al., 1994) and is performed using microinjection of native mRNA or in vitro synthesized mRNA into frog oocytes. The preparation of in  
15 vitro synthesized mRNA can be performed by various standard techniques (Sambrook, et al. 1989) including using T7 polymerase with the mCAP RNA mapping kit (Stratagene).

20 This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor agonist effective to treat the abnormality. In separate  
25 embodiments, the abnormality is a lower urinary tract disorder such as interstitial cystitis or urinary incontinence such as urge incontinence or stress incontinence particularly stress incontinence, a regulation of a steroid hormone disorder, an epinephrine release  
30 disorder, a gastrointestinal disorder, irritable bowel syndrome, a cardiovascular disorder, an electrolyte balance disorder, diuresis, hypertension, hypotension, diabetes, hypoglycemia, a respiratory disorder, asthma, a reproductive function disorder, an immune disorder, an endocrine  
35 disorder, a musculoskeletal disorder, a neuroendocrine disorder, a cognitive disorder, a memory disorder, a sensory modulation and transmission disorder, a motor coordination disorder, a sensory integration disorder, a motor

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integration disorder, a dopaminergic function disorder, a serotonergic function disorder, an appetite disorder, obesity, a sensory transmission disorder, an olfaction disorder, a sympathetic innervation disorder, an affective disorder, pain, psychotic behavior, morphine tolerance, nicotine addiction, opiate addiction, or migraine.

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor antagonist effective to treat the abnormality. In separate embodiments, the abnormality is a lower urinary tract disorder such as interstitial cystitis or urinary incontinence such as urge incontinence or stress incontinence particularly stress incontinence, a regulation of a steroid hormone disorder, an epinephrine release disorder, a gastrointestinal disorder, irritable bowel syndrome, a cardiovascular disorder, an electrolyte balance disorder, diuresis, hypertension, hypotension, diabetes, hypoglycemia, a respiratory disorder, asthma, a reproductive function disorder, an immune disorder, an endocrine disorder, a musculoskeletal disorder, a neuroendocrine disorder, a cognitive disorder, a memory disorder, a sensory modulation and transmission disorder, a motor coordination disorder, a sensory integration disorder, a motor integration disorder, a dopaminergic function disorder, a serotonergic function disorder, an appetite disorder, obesity, a sensory transmission disorder, an olfaction disorder, a sympathetic innervation disorder, an affective disorder, pain, psychotic behavior, morphine tolerance, nicotine addiction, opiate addiction, or migraine.

This invention also provides the use of mammalian NPFF receptors for analgesia.

This invention provides a process for making a composition

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of matter which specifically binds to a mammalian NPFF receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. In one embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF2 receptor.

This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor or a novel structural and functional analog or homolog thereof. In one embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF2 receptor.

Thus, once the gene for a targeted receptor subtype is cloned, it is placed into a recipient cell which then expresses the targeted receptor subtype on its surface. This cell, which expresses a single population of the targeted human receptor subtype, is then propagated resulting in the establishment of a cell line. This cell line, which constitutes a drug discovery system, is used in two different types of assays: binding assays and functional assays. In binding assays, the affinity of a compound for both the receptor subtype that is the target of a particular drug discovery program and other receptor subtypes that could be associated with side effects are measured. These measurements enable one to predict the potency of a compound, as well as the degree of selectivity that the compound has for the targeted receptor subtype over other

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receptor subtypes. The data obtained from binding assays also enable chemists to design compounds toward or away from one or more of the relevant subtypes, as appropriate, for optimal therapeutic efficacy. In functional assays, the nature of the response of the receptor subtype to the compound is determined. Data from the functional assays show whether the compound is acting to inhibit or enhance the activity of the receptor subtype, thus enabling pharmacologists to evaluate compounds rapidly at their ultimate human receptor subtypes targets permitting chemists to rationally design drugs that will be more effective and have fewer or substantially less severe side effects than existing drugs.

Approaches to designing and synthesizing receptor subtype-selective compounds are well known and include traditional medicinal chemistry and the newer technology of combinatorial chemistry, both of which are supported by computer-assisted molecular modeling. With such approaches, chemists and pharmacologists use their knowledge of the structures of the targeted receptor subtype and compounds determined to bind and/or activate or inhibit activation of the receptor subtype to design and synthesize structures that will have activity at these receptor subtypes.

Combinatorial chemistry involves automated synthesis of a variety of novel compounds by assembling them using different combinations of chemical building blocks. The use of combinatorial chemistry greatly accelerates the process of generating compounds. The resulting arrays of compounds are called libraries and are used to screen for compounds ("lead compounds") that demonstrate a sufficient level of activity at receptors of interest. Using combinatorial chemistry it is possible to synthesize "focused" libraries of compounds anticipated to be highly biased toward the receptor target of interest.

Once lead compounds are identified, whether through the use

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of combinatorial chemistry or traditional medicinal chemistry or otherwise, a variety of homologs and analogs are prepared to facilitate an understanding of the relationship between chemical structure and biological or functional activity. These studies define structure activity relationships which are then used to design drugs with improved potency, selectivity and pharmacokinetic properties. Combinatorial chemistry is also used to rapidly generate a variety of structures for lead optimization. Traditional medicinal chemistry, which involves the synthesis of compounds one at a time, is also used for further refinement and to generate compounds not accessible by automated techniques. Once such drugs are defined the production is scaled up using standard chemical manufacturing methodologies utilized throughout the pharmaceutical and chemistry industry.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

## EXPERIMENTAL DETAILS

### Materials and methods

#### 5 Cloning of rat and human NPFF1 receptor

##### MOPAC (Mixed Oligonucleotide Primed Amplification of cDNA

100ng of rat genomic DNA (Clontech, Palo Alto, CA) was used for degenerate MOPAC PCR using Taq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN) and the following  
10 degenerate oligonucleotides: JAB126, designed based on an alignment of the sixth transmembrane domain of more than 180 members of the rhodopsin superfamily of G protein-coupled receptors; and JAB108, designed based on an alignment of the  
seventh transmembrane domain of the same rhodopsin  
15 superfamily.

The conditions for the MOPAC PCR reaction were as follows:  
3 minute hold at 94°C; 10 cycles of 1 minute at 94°C, 1  
minute 45 seconds at 44°C, 2 minutes at 72°C; 30 cycles of  
20 94°C for 1 minute, 49°C for 1 minute 45 seconds, 2 minutes  
at 72°C; 4 minute hold at 72°C; 4°C until ready for agarose  
gel electrophoresis.

The products were run on a 1% agarose TAE gel and bands of  
25 the expected size (~150bp) were cut from the gel, purified  
using the QIAQUICK gel extraction kit (QIAGEN, Chatsworth,  
CA), and subcloned into the TA cloning vector (Invitrogen,  
San Diego, CA). White (insert-containing) colonies were  
picked and subjected to PCR using pCR2.1 vector primers JAB1  
30 and JAB2 using the Expand Long Template PCR System and the  
following protocol: 94°C hold for 3 minutes; 35 cycles of  
94°C for 1 minute, 68°C for 1 minute 15 seconds; 2 minute  
hold at 68°C, 4°C hold until products were ready for  
purification. PCR products were purified by isopropanol  
35 precipitation (10 µl PCR product, 18 µl low TE, 10.5 µl 2M  
NaClO<sub>4</sub> and 21.5 µl isopropanol) and sequenced using the ABI  
Big Dye cycle sequencing protocol and ABI 377 sequencers  
(ABI, Foster City, CA). Nucleotide and amino acid sequence

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analyses were performed using the Wisconsin Package (GCG, Genetics Computer Group, Madison, WI). Two PCR products produced from rat genomic cDNA (MPR3-RGEN-31 and MPR3-RGEN-45) were determined to be identical clones of a novel G protein-coupled receptor-like sequence based on database searches and its homology to other known G protein-coupled receptors (~30-40% amino acid identity to dopamine D<sub>2</sub>, orexin, galanin, angiotensin 1 and 5-HT<sub>2b</sub> receptors). This novel sequence was designated SNORF2.

Cloning of the full-length coding sequence of SNORF2 (rat NPFF1)

Pools of the rat hypothalamic cDNA library "I" were screened by PCR with SNORF2-specific primers JAB208 and JAB209 and the Expand Long Template PCR system (Boehringer-Mannheim, Indianapolis, IN) with the following PCR protocol: 94°C hold for 3 minutes; 40 cycles of 94°C for 1 minute, 68°C for 2 minutes; 4 minute hold at 68°C; 4°C hold until the samples are run on a gel. This screen yielded a positive pool I36E and a positive sub-pool I36E-17. High stringency hybridization of isolated colonies from I36E-17 with the SNORF2-specific oligonucleotide probe JAB211 and subsequent PCR testing of positive colonies indicated that the isolated clone I36E-17-1B-1 contained at least a partial clone of SNORF2. Sequencing of I36E-17-1B-1 revealed that this insert contained the coding region from the TMIII-TMIV loop through the stop codon, including some 3' untranslated sequence. From this sequence, a new forward primer, JAB221, was designed in TMV. PCR screening of a second rat hypothalamic cDNA library "J" with primers JAB221 and JAB209, and subsequent colony hybridization with the JAB211 probe on a low complexity positive sub-pool resulted in the isolation of a SNORF2 clone J-13-16-A1. Full-length double-stranded sequence of SNORF2 was determined by sequencing both strands of the J-13-16-A1 plasmid using an ABI 377 sequencer as described above. This insert is about 2.8 kb in length with an approximately 200 bp 5' untranslated region, a 1296 bp coding region, and a 1.3 kb 3'untranslated region. The clone

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is also in the correct orientation for expression in the mammalian expression vector pEXJ.T7. This construct of SNORF2 in pEXJ.T7 was designated BN-6. The full length SNORF2 was determined to be most like the orexin 1 receptor (45% DNA identity, 35% amino acid identity), orexin 2 receptor (40% DNA identity, 32% amino acid identity), and NPY2 receptor (47% DNA identity, 29% amino acid identity), although several other G protein-coupled receptors also displayed significant homology. There were no sequences in the Genbank databases (genembl, sts, est, gss, or swissprot) that were identical to SNORF2. SNORF2 also showed significant homology (85% nucleotide identity, 93% amino acid identity) to a partial G protein-coupled receptor fragment in the Synaptic Pharmaceutical Corporation in-house database, designated PLC29b. PLC29b, which includes part of the amino terminus through TMIII, was originally isolated from a human genomic library using oligonucleotide probes for NPY4. Subsequent screening of a human hippocampal cDNA library yielded an overlapping sequence extending into TMIV. Based on sequence similarity, this human sequence appears to be a partial clone of the human homolog of SNORF2.

The following is a list of primers and their associated sequences which were used in the cloning of these receptors:

JAB126: 5'-GYNTWYRYNNTNWSNTGGHTNCC-3' (SEQ ID NO: 9)  
JAB108: 5'-AVNADNGBRWAVANNANNGGRTT-3' (SEQ ID NO: 10)  
JAB1: 5'-TTATGCTTCCGGCTCGTATGTTGTG-3' (SEQ ID NO: 11)  
JAB2: 5'-ATGTGCTGCAAGGCGATTAAGTTGGG-3' (SEQ ID NO: 12)  
JAB208: 5'-GGTGCTGCTGCTGCTCATCGACTATG-3' (SEQ ID NO: 13)  
JAB209: 5'-TTGGCGCTGCTGTGGAAGAAGGCCAG-3' (SEQ ID NO: 14)  
JAB221: 5'-CGGTGCTCTTCGCGCACATCTACC-3' (SEQ ID NO: 15)  
JAB211: 5'-TGCCAAGGGGAAGGCGTAGACCGACAGCAGGTGCAGTTGCA  
GCTCGATCAGCTCCCCATA-3' (SEQ ID NO: 16)

Isolation of the full-length human SNORF2 receptor gene (human NPFF1)

The full-length, intronless version of the human NPFF1



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receptor gene may be isolated using standard molecular biology techniques and approaches such as those briefly described below:

5      Approach #1: To obtain a full-length human NPFF1 receptor, a human cosmid library was screened with a <sup>32</sup>P-labeled oligonucleotide probe, BB609, corresponding to the 2/3 loop of the PLC29b clone. A positive clone was isolated and partially sequenced, revealing part of the amino terminus and TMs I and II.

10      The full-length sequence may be obtained by sequencing this cosmid clone with additional sequencing primers. Since at least two introns are present in this gene, one in the amino terminus and one just after the third transmembrane domain, the full-length intronless gene may be obtained from cDNA using standard molecular biology techniques. For example, a forward PCR primer designed in the 5'UT and a reverse PCR primer designed in the 3'UT may be used to amplify a full-length, intronless gene from cDNA. RT-PCR localization has identified several human tissues which could be used for this purpose, including cerebellum, spinal cord, hippocampus, lung and kidney. Standard molecular biology techniques could be used to subclone this gene into a mammalian expression vector.

25      Approach #2: Standard molecular biology techniques could be used to screen commercial human cDNA phage libraries by hybridization under high stringency with a <sup>32</sup>P-labeled oligonucleotide probe, BB609, corresponding to the 2/3 loop of the PLC29b clone. One may isolate a full-length human NPFF1 by obtaining a plaque purified clone from the lambda libraries and then subjecting the clone to direct DNA sequencing using primers from the PLC29b sequence.

30      Alternatively, standard molecular biology techniques could be used to screen in-house human cDNA plasmid libraries by PCR amplification of library pools using primers to the human NPFF1 sequence (BB629, forward primer in TMI, and A71,

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reverse primer in TMIV). A full-length clone could be isolated by Southern hybridization of colony lifts of positive pools with a <sup>32</sup>P-labeled oligonucleotide probe, BB609, corresponding to the 2/3 loop of the PLC29b clone.

5

Approach #3: As yet another alternative method, one could utilize 3' and 5' RACE to generate PCR products from human cDNA expressing human NPFF1 (for example, cerebellum, spinal cord, hippocampus, lung and kidney), which contain the additional sequences of human NPFF1. For 5' RACE, a reverse primer derived from PLC29b between the amino terminus and TM IV could be used to amplify the additional amino terminus sequence for hNPFF1. For 3' RACE, a forward primer derived from PLC29b between the amino terminus and TM IV could be used to amplify the additional 3' sequence for hNPFF1, including TMs 5-7 and the COOH terminus. These RACE PCR product could then be sequenced to determine the missing sequence. This new sequence could then be used to design a forward PCR primer in the 5'UT and a reverse primer in the 3'UT. These primers could then be used to amplify a full-length hNPFF1 clone from human cDNA sources known to express NPFF1 (for example, cerebellum, spinal cord, hippocampus, lung and kidney).

25 BB609: 5'-CCACCCTTGTGGACAACCTCATCACTGGGTGGCCCTTCGACA  
ATGCCACATGC-3' (SEQ ID NO: 17)

BB629: 5'-CTGCTCTGCATGGTGGGCAACACC-3' (SEQ ID NO: 18)

30 A71: 5'-GACGGCGATGGTGACGAGCGC-3' (SEQ ID NO: 19)

#### Cloning of human NPFF1 receptor

35 The sequence of the human NPFF1 (hNPFF1) receptor from the initiating methionine to TMIV was determined to be present in a partial clone, plc29b, found in a Synaptic Pharmaceutical Corporation in-house database. In order to isolate the full-length hNPFF1 receptor cDNA, a human cosmid

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library (Stratagene) was screened with a <sup>32</sup>P-labeled probe (BB609) corresponding to the II/III loop of plc29b. Partial DNA sequencing of one positive clone from this library, COS28a revealed similar sequence as had been previously shown for plc29b, with an intron downstream of TMIII. In order to obtain sequence in the 3' end of hNPFF1, COS28a was amplified with a vector primer and BB702, BB703 or BB704, forward primers in TMIV. DNA sequencing of these PCR products resulted in the identification of TMIV through the stop codon.

Next, an in-house human spinal cord library was screened by PCR using a forward primer in the region of the initiating methionine (BB729) and a reverse primer corresponding to TMIV (BB728). One positive pool, W4, was subdivided and a positive sub-pool was screened by colony hybridization with a <sup>32</sup>P-labeled probe from TMII, BB676. Plasmid DNA was isolated for clone W4-18-4, renamed BO98, and DNA sequencing revealed that it was full-length but in the wrong orientation for expression in the expression vector pEXJ. To obtain a full-length hNPFF1 construct in the correct orientation, BO98 was amplified with BB757, a forward primer at the initiating methionine which contained an upstream BamHI site, and BB758, a reverse primer at the stop codon which contained a EcoRI site. The products from 3 independent PCR reactions were ligated into pcDNA3.1+ and transformed into DH5α cells. The sequence of one of these transformants, 3.3, was identical to the hNPFF1 sequence previously determined from the consensus of BO98, COS28a and plc29b. Clone 3.3 was renamed BO102.

The hNPFF1 clone contains an open reading frame with 1293 nucleotides and predicts a protein of 430 amino acids (Figures 11 and 12). Hydrophobicity analysis reveals seven hydrophobic domains which are presumed to be transmembrane domains (Figure 13). The sequence of hNPFF1 was determined to be most similar to the rat NPFF1 (86% nucleotide identity, 87% amino acid identity) and human NPFF2 (56%

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nucleotide identity, 49% amino acid identity (Figure 14)). The human NPFF1 receptor also shares homology with human orexin<sub>1</sub> (53% nucleotide identity, 35% amino acid identity), human orexin<sub>2</sub> (43% nucleotide identity, 33% amino acid identity), human NPY<sub>2</sub> (47% nucleotide identity, 31% amino acid identity), human CCK<sub>A</sub> (46% nucleotide identity, 32% amino acid identity), and human CCK<sub>B</sub> (46% nucleotide identity, 26% amino acid identity).

The following primers and probes were used in the cloning of hNPFF1:

BB676: 5'-GTCACCAACATGTTTCATCCTCAACCTGGCTGTCAGTGACCTGCT  
GGTGGGCATCTTCTGCATGCC-3' (SEQ ID NO: 20)  
BB702: 5'-GCGAGAAGCTGACCCTGCGGAAGG-3' (SEQ ID NO: 21)  
BB703: 5'-TCGTCACCATCGCCGTCATCTGGG-3' (SEQ ID NO: 22)  
BB704: 5'-CGTCATCTGGGCCGAGGGACACAG-3' (SEQ ID NO: 23)  
BB728: 5'-TGACGGCGATGGTGACGAGCGCC-3' (SEQ ID NO: 24)  
BB729: 5'-CAGCCTCCCAACAGCAGTTGGCC-3' (SEQ ID NO: 25)  
BB757: 5'-TAGCAAGGATCCGCATATGGAGGGGAGCCCTCCC-3' (SEQ  
ID NO: 26)  
BB758: 5'-CTTCATGAATTCATCGCCTGCATGTATCTCGTGTCC-3' (SEQ  
ID NO: 27)

#### Cloning of human NPFF2 receptor

Discovery of an expressed sequence tag (EST) AA449919 in GENEMBL homologous to rNPFF1 (hNPFF2)

A FASTA search of GENEMBL with the full-length sequence of rat NPFF1 (rNPFF1) resulted in the identification of an EST (Accession number AA449919) with a high degree of homology to NPFF1 (57% identity at the DNA level). AA449919 is a 532 bp sequence annotated in Genbank as "Soares total fetus Nb2HF8 9w Homo sapiens cDNA clone 788698 5' similar to SW:NYR\_DROME P25931 NEUROPEPTIDE Y RECEPTOR," which when translated corresponds to the region between the first extracellular loop and the beginning of the sixth transmembrane domain of rNPFF1. GAP analysis of AA449919

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with rNPFF1 indicated that there is 57% DNA identity and a 50% amino acid identity between the two receptor sequences over this region. AA449919 displays 60% DNA identity and 59% amino acid identity over the region that overlaps with the known sequence for hNPFF1 (first extracellular loop to TM4), while over the same range rNPFF1 is 62% and 61% identical to AA449919 at the DNA and amino acid levels, respectively. In comparison, hNPFF1 and rNPFF1 share 86% DNA identity and 92 % amino acid identity over this region. Given the strong degree of identity between AA449919 and rNPFF1, AA449919 was given the name NPFF-like (hNPFF2).

Cloning the full-length sequence of (NPFF-like) hNPFF2

To determine the full-length coding sequence of AA449919, 5'/3' Rapid Amplification of cDNA ends (RACE) was performed on Clontech Human Spleen Marathon-Ready cDNA (Clontech, Palo Alto, CA). For 5' RACE, 5 $\mu$ l template (human spleen Marathon-Ready cDNA was amplified with oligonucleotide primers JAB256 and AP1, the Expand Long DNA Template PCR System (Boehringer-Mannheim, Indianapolis, IN) and the following PCR protocol were used: 94°C hold for 3 minutes; 5 cycles of 94°C for 30 seconds, 72°C for 4 minutes; 5 cycles of 94°C for 30 seconds, 70°C for 4 minutes; 30 cycles of 94°C for 30 seconds, 68°C for 4 minutes; 68°C hold for 4 minutes; 4°C hold until products were ready to be loaded on a gel. 1 $\mu$ l of this reaction was subjected to a second round of amplification with primers JAB260 and AP2 and the same PCR protocol. For 3' RACE, 5  $\mu$ l human spleen Marathon-Ready cDNA was subjected to PCR with primers JAB257 and AP1 with the same PCR protocol that was used for 5' RACE. 1 $\mu$ l of this reaction was subjected to another round of amplification using AP2 and JAB258 and the same PCR conditions.

The products were run on a 1% agarose TAE gel and bands greater than 500 bp were extracted from the gel using the QIAQUICK gel extraction kit (QIAGEN, Chatsworth, CA). 5  $\mu$ l of each purified band from the 5' and 3' RACE reactions were

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directly sequenced with primers JAB261 (5' products) and JAB259 (3' products) using the ABI Big Dye cycle sequencing protocol and ABI377 sequencers (ABI, Foster City, CA). The Wisconsin Package (GCG, Genetics Computer Group, Madison, WI) and Sequencer 3.0 (Gene Codes Corporation, Ann Arbor, MI) were used to put together the full-length contiguous sequence of hNPFF2 from the AA449919 EST and the RACE products.

To attain the full-length hNPFF-like (hNPFF2) coding sequence for expression, human spinal cord cDNA was amplified in eight independent PCR reactions using the Expand Long Template PCR System with buffer I (four of the eight reactions) or buffer 3 (4 reactions) and two oligonucleotide primers with restriction sites incorporated into their 5' ends: BB675 is a forward primer upstream of the initiating methionine and contains a BamHI site, and BB663. The PCR conditions for this reaction were as follows: 94°C hold for 5 minutes; 37 cycles of 94°C for 30 seconds, 64°C for 30 seconds, 68°C for 2 minutes; a 7 minute hold at 68°C, and a 4°C hold until products were ready to be loaded on a gel. The products were electrophoresed on a 1% agarose TAE gel, and a band of approximately 1.35 kb was cut and purified using the QIAQUICK gel extraction kit. The purified bands of seven of the eight reactions were cut with BamHI and EcoRI, gel purified again using the same method, and ligated into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Eighteen colonies from the subsequent transformations were picked and determined to be positive for NPFF-like by PCR. Eight of these 18 clones were fully sequenced, and one of these, BO89, was determined to be a full length clone with no point mutations. This construct was renamed pcDNA3.1-hNPFF2b.

For expression of NPFF-like in oocytes, one ul of each of these eight ligations of the BB675-BB663 PCR product into pcDNA3.1(+) was subjected to PCR with AN35, a pcDNA3.1 primer at the CMV promoter site, and the 3' NPFF-like primer

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BB663 using the Expand Long Template PCR System and the following PCR protocol: 94°C hold for 3 minutes; 37 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 68°C for 2 minutes; a 7 minute hold at 68°C, and a 4°C hold until products were ready for in vitro transcription. Of the seven PCR reactions, six yielded products of the expected size.

The following is a list of primers and their associated sequences which were used in the cloning of this receptor (hNPFF2):

AN35: 5'-CGTGTACGGTGGGAGGTCTATATAAGCAGAG-3' (SEQ ID NO: 28)

15 AP1: 5'-CCATCCTAATACGACTCACTATAGGGC-3' (SEQ ID NO: 29)

AP2: 5'-ACTCACTATAGGGCTCGAGCGGC-3' (SEQ ID NO: 30)

JAB256: 5'-TGATAGTGAGCTTTGGTTTAAAGGG-3' (SEQ ID NO: 31)

JAB257: 5'-GAAGATCTACACCACTGTGCTGTTTG-3' (SEQ ID NO: 32)

JAB258: 5'-AACATCTACCTGGCTCCCCTCTCCC-3' (SEQ ID NO: 33)

20 JAB259: 5'-TTGTCATCATGTATGGAAGGATTGG-3' (SEQ ID NO: 34)

JAB260: 5'-GACCACACACTGGAACCTATCTAC-3' (SEQ ID NO: 35)

JAB261: 5'-GCAATTGCAACTAACGTAAAGACTG-3' (SEQ ID NO: 36)

BB675: 5'-TAGCAAGGATCCGAGGTTTCATCATGAATGAGAAATGG-3' (SEQ ID NO: 37)

25 BB663: 5'-CTTCATGAATTCGCGTAGTAGAGTTAGGATTATCAC-3' (SEQ ID NO: 38)

For expression of NPFF2, mRNA transcripts were generated as described for NPFF1, using PCR products from ligation reactions or linearized DNA from BO89 as DNA templates. Oocytes were injected with 5-50 ng NPFF2 mRNA and incubated as previously described.

#### Isolation of the Rat Homologue of NPFF2

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To obtain a fragment of the rat homologue of NPFF2, rat genomic DNA (Clontech, Palo Alto, CA), rat hypothalamic cDNA or rat spinal cord cDNA was amplified with a forward PCR

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primer corresponding to TMIV of human NPFF2 (JAB307) and a reverse primer corresponding to TMVI of human NPFF2 (JAB 306). PCR was performed with the Expand Long Template PCR System (Roche Molecular Biochemicals, Indianapolis, IN) under the following conditions: 1 minute at 94°C, 2 minutes at 50°C, 2 minutes at 68°C for 40 cycles, with a pre- and post-incubation of 3 minutes at 94°C and 4 minutes at 68°C respectively. Bands of 368 bp from 3 independent PCR reactions were isolated from a TAE gel, purified using the QIAQUICK gel extraction kit (QIAGEN, Chatsworth, CA), and sequenced on both strands as described above. The sequences of these 3 PCR products were identical.

To obtain additional sequence for rat NPFF2, reduced stringency PCR was performed using primers designed against the human NPFF2 NH<sub>2</sub> and COOH termini along with PCR primers designed against the rat NPFF2 fragment. For the NH<sub>2</sub> terminal sequence, PCR was performed on rat spinal cord cDNA with BB665, a sense primer just upstream of TMI in human NPFF2, and BB795, an antisense primer in the second extracellular loop of the rat NPFF2. For the COOH terminal sequence, PCR was performed on rat spinal cord cDNA with BB793, a sense primer from the third intracellular loop in rat NPFF2, and BB668, an antisense primer just downstream from TMVII in human NPFF2. PCR was performed using the Expand Long Template PCR System (Roche Biochemicals, Indianapolis, IN) with buffer 2 (NH<sub>2</sub> terminal) or buffer 1 (COOH terminal) and the following conditions: 30 seconds at 94°C, 30 seconds at 42°C (NH<sub>2</sub> terminal) or 50°C (COOH terminal), 1.5 minutes at 68°C for 40 cycles, with a pre- and post-incubation of 3 minutes at 94°C and 4 minutes at 68°C respectively. A 500 bp band from the NH<sub>2</sub> terminal PCR and a 300 bp band from the COOH terminal PCR were isolated from a TAE gel, purified using the QIAQUICK gel extraction kit (QIAGEN, Chatsworth, CA), and sequenced on both strands as described above.

A rat liver genomic phage library (2.75 million



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recombinants, Stratagene, LaJolla, CA) was screened using a  $^{32}\text{P}$ -labeled oligonucleotide probe, BB712, corresponding to the second extracellular loop and TMV of the rat NPFF2 fragment above. Hybridization of nitrocellulose filter overlays of the plates was performed at high stringency: 42°C in a solution containing 50% formamide, 5x SSC (1X SSC is 0.15M sodium chloride, 0.015M sodium citrate), 1x Denhardt's solution (0.02% polyvinylpyrrolindone, 0.02% Ficoll, 0.02% bovine serum albumin), 7 mM Tris and 25 µg/ml sonicated salmon sperm DNA. The filters were washed at 55°C in 0.1x SSC containing 0.1% sodium dodecyl sulfate and exposed at -70°C to Kodak BioMax MS film in the presence of an intensifying screen.

Three positive signals, rNPFF2-1, rNPFF2-4 and rNPFF2-6 were isolated on a tertiary plating. A 3.5 kb fragment, from a BglII/EcoRI digest of DNA isolated from rNPFF2-6, was identified by Southern blot analysis with BB712, subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and used to transform E. coli DH5α cells (Gibco BRL, Gaithersburg MD). Plasmid DNA from one transformant was sequenced using an ABI 377 sequencer as described above. Sequencing with HK137, a sense primer from TMV of the rat NPFF2 fragment revealed the sequence for TMVII, the COOH terminus and some 3'UT. Sequencing with HK139, an antisense primer from TMII of rNPFF2, revealed the presence an intron upstream of TMII.

To determine if any of the three positive plaques contained sequence upstream of this intron, DNA from each of the clones were spotted onto nitrocellulose and hybridized with HK140, an oligonucleotide probe corresponding to TMI of the rat NPFF2 fragment. The rNPFF2-1 and rNPFF2-4 clones were positive. A 2.1 kb fragment, from a HindIII digest of DNA isolated from rNPFF2-4, was identified by Southern blot analysis with HK140, subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and used to transform E. coli DH5α cells (Gibco BRL, Gaithersburg MD). Sequencing of this fragment with HK138, an antisense primer from TMI of rat NPFF2,

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revealed the NH<sub>2</sub> terminus and 5'UT of the rat NPFF2 receptor.

The full-length NPFF2 was amplified from rat spinal cord cDNA using a sense primer in the 5'UT (HK146, also incorporating a BamHI restriction site) and an antisense primer from the 3'UT (HK147, also incorporating a BstXI restriction site) and the Expand Long Template PCR System (Roche Molecular Biochemicals, Indianapolis, IN) using buffer 2 and the following PCR conditions: 30 seconds at 94°C, 2.5 minutes at 68°C for 32 cycles, with a pre- and post-incubation of 5 minutes at 94°C and 7 minutes at 68°C, respectively. Products from 5 independent PCR reactions were gel-purified. 1 µl of each reaction was used as a template to re-amplify the product using the same PCR conditions. The products were digested with BamHI and BstXI and ligated into a modified pcDNA3.1 vector (Invitrogen, San Diego, CA). Products from each PCR reaction were sequenced as above. While a consensus amino acid sequence was determined among the PCR products, there was some ambiguity in the nucleotide sequence at 4 positions. To determine if this represented PCR-induced errors or allelic variations, the areas in question were amplified from several lots of genomic DNA. Sequencing of these genomic products revealed the same ambiguities, suggesting allelic variations at these residues. One construct, KO31, was renamed BO119 and later renamed pcDNA3.1-rNPFF2-f.

Oligonucleotide primers and probes used in the identification and isolation of the rat NPFF2:

JAB307: 5'-TTTGTCATTATTATGATCATCTGG-3' (SEQ ID NO: 47)  
JAB306: 5'-AATAAAAAGCAGGGCCACAATCAG-3' (SEQ ID NO: 48)  
BB665: 5'-TCATTATTTCTACTTTCTGATC-3' (SEQ ID NO: 49)  
BB795: 5'-CTCATTTCTGGTTTGGCCAATCC-3' (SEQ ID NO: 50)  
BB793: 5'-TCTTCAAGACCTCAGCACACAGC-3' (SEQ ID NO: 51)  
BB668: 5'-GAGCTGGAAAGCTTCTTGGAAC-3' (SEQ ID NO: 52)  
BB712: 5'-CTGGTGTCTGGGAGGATTGGCCAAACCAGGAAATGAGGAGGAT  
CTACACC-3' (SEQ ID NO: 53)

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HK137: 5'-GCAGTGTCAACCCCATCATTTATGG-3' (SEQ ID NO: 54)

HK138: 5'-CAAAGCAAACGACAGTGTTCACACC-3' (SEQ ID NO: 55) HK139:  
5'-AGTGACCGTGTGCATGTACCTATTCC-3' (SEQ ID NO: 56)

HK140: 5'-GGTGGGAAACACTGTCGTTTGCTTTGTTGTAATAAGGAATA

5 GGTACATGCACACGGTCAC -3' (SEQ ID NO: 57)

HK146: 5'-GTCACGGATCCAGCCTCTCCTTTGATAAGGTCCACC -3'  
(SEQ ID NO: 58)

HK147: 5'-GTCAGCCATCGAGTTGGCTTCGTATGCTATATAACA  
TTGGATAGC-3' (SEQ ID NO: 59)

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Isolation of other species homologs of NPFF1 or NPFF2  
receptor cDNA

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A nucleic acid sequence encoding a NPFF1 or NPFF2 receptor  
cDNA from other species may be isolated using standard  
molecular biology techniques and approaches such as those  
described below:

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Approach #1: A genomic library (e.g., cosmid, phage, P1,  
BAC, YAC) generated from the species of interest may be  
screened with a <sup>32</sup>P-labeled oligonucleotide probe  
corresponding to a fragment of the human or rat NPFF1 or  
NPFF2 receptors whose sequence is shown in Figures 1, 7, 11  
and 22A-C to isolate a genomic clone. The full-length  
sequence may be obtained by sequencing this genomic clone.  
If one or more introns are present in the gene, the  
full-length intronless gene may be obtained from cDNA using  
standard molecular biology techniques. For example, a  
forward PCR primer designed in the 5'UT and a reverse PCR  
primer designed in the 3'UT may be used to amplify a  
full-length, intronless receptor from cDNA. Standard  
molecular biology techniques could be used to subclone this  
gene into a mammalian expression vector.

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Approach #2: Standard molecular biology techniques may be  
used to screen commercial cDNA phage libraries of the  
species of interest by hybridization under reduced  
stringency with a <sup>32</sup>P-labeled oligonucleotide probe

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corresponding to a fragment of the sequences shown in Figures 1, 7, 11 and 22A-C. One may isolate a full-length NPFF1 or NPFF2 receptor by obtaining a plaque purified clone from the lambda libraries and then subjecting the clone to direct DNA sequencing. Alternatively, standard molecular biology techniques could be used to screen cDNA plasmid libraries by PCR amplification of library pools using primers designed against a partial species homolog sequence. A full-length clone may be isolated by Southern hybridization of colony lifts of positive pools with a <sup>32</sup>P-labeled oligonucleotide probe.

Approach #3: 3' and 5' RACE may be utilized to generate PCR products from cDNA derived from the species of interest expressing NPFF1 or NPFF2 which contain the additional sequence of NPFF1 or NPFF2. These RACE PCR products may then be sequenced to determine the additional sequence. This new sequence is then used to design a forward PCR primer in the 5'UT and a reverse primer in the 3'UT. These primers are then used to amplify a full-length NPFF1 or NPFF2 clone from cDNA.

Examples of other species include, but are not limited to, mouse, dog, monkey, hamster and guinea pig.

#### Cell culture

COS-7 cells are grown on 150 mm plates in DMEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 µg/ml streptomycin) at 37°C, 5% CO<sub>2</sub>. Stock plates of COS-7 cells are trypsinized and split 1:6 every 3-4 days.

Human embryonic kidney 293 cells (HEK-293 cells) are grown on 150 mm plates in DMEM with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 µg/ml streptomycin) at 37°C, 5% CO<sub>2</sub>. Stock plates of 293 cells are trypsinized and split 1:6 every 3-4 days.

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5 Mouse fibroblast LM(tk-) cells are grown on 150 mm plates in D-MEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100  $\mu$ g/ml streptomycin) at 37°C, 5% CO<sub>2</sub>. Stock plates of LM(tk-) cells are trypsinized and split 1:10 every 3-4 days.

10 Chinese hamster ovary (CHO) cells were grown on 150 mm plates in HAM's F-12 medium with supplements (10% bovine calf serum, 4 mM L-glutamine and 100 units/ml penicillin/ 100  $\mu$ g/ml streptomycin) at 37°C, 5% CO<sub>2</sub>. Stock plates of CHO cells are trypsinized and split 1:8 every 3-4 days.

15 Mouse embryonic fibroblast NIH-3T3 cells are grown on 150 mm plates in Dulbecco's Modified Eagle Medium (DMEM) with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100  $\mu$ g/ml streptomycin) at 37°C, 5% CO<sub>2</sub>. Stock plates of NIH-3T3 cells are trypsinized and split 1:15 every 3-4 days.

20 Sf9 and Sf21 cells are grown in monolayers on 150 mm tissue culture dishes in TMN-FH media supplemented with 10% fetal calf serum, at 27°C, no CO<sub>2</sub>. High Five insect cells are grown on 150 mm tissue culture dishes in Ex-Cell 400™ medium  
25 supplemented with L-Glutamine, also at 27°C, no CO<sub>2</sub>.

#### Transient transfection

Receptors studied may be transiently transfected into COS-7 cells by the DEAE-dextran method using 1  $\mu$ g of DNA /10<sup>6</sup> cells  
30 (Cullen, 1987). In addition, Schneider 2 Drosophila cells may be cotransfected with vectors containing the receptor gene under control of a promoter which is active in insect cells, and a selectable resistance gene, eg., the G418 resistant neomycin gene, for expression of the polypeptides  
35 disclosed herein.

#### Stable transfection

DNA encoding the human receptor disclosed herein may be

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co-transfected with a G-418 resistant gene into the human embryonic kidney 293 cell line by a calcium phosphate transfection method (Cullen, 1987). Stably transfected cells are selected with G-418.

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#### Membrane preparations

LM(tk-) cells stably transfected with the DNA encoding the human receptor disclosed herein may be routinely converted from an adherent monolayer to a viable suspension. Adherent  
10 cells are harvested with trypsin at the point of confluence, resuspended in a minimal volume of complete DMEM for a cell count, and further diluted to a concentration of  $10^6$  cells/ml in suspension media (10% bovine calf serum, 10% 10X Medium 199 (Gibco), 9 mM  $\text{NaHCO}_3$ , 25 mM glucose, 2 mM L-glutamine,  
15 100 units/ml penicillin/100  $\mu\text{g/ml}$  streptomycin, and 0.05% methyl cellulose). Cell suspensions are maintained in a shaking incubator at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  for 24 hours. Membranes harvested from cells grown in this manner may be stored as large, uniform batches in liquid nitrogen. Alternatively,  
20 cells may be returned to adherent cell culture in complete DMEM by distribution into 96-well microtiter plates coated with poly-D-lysine (0.01 mg/ml) followed by incubation at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  for 24 hours.

#### Generation of baculovirus

The coding region of DNA encoding the human receptors disclosed herein may be subcloned into pBlueBacIII into existing restriction sites or sites engineered into sequences 5' and 3' to the coding region of the  
30 polypeptides. To generate baculovirus, 0.5  $\mu\text{g}$  of viral DNA (BaculoGold) and 3  $\mu\text{g}$  of DNA construct encoding a polypeptide may be co-transfected into  $2 \times 10^6$  Spodoptera frugiperda insect Sf9 cells by the calcium phosphate co-precipitation method, as outlined by Pharmingen (in  
35 "Baculovirus Expression Vector System: Procedures and Methods Manual"). The cells then are incubated for 5 days at  $27^\circ\text{C}$ .

The supernatant of the co-transfection plate may be

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collected by centrifugation and the recombinant virus plaque purified. The procedure to infect cells with virus, to prepare stocks of virus and to titer the virus stocks are as described in Pharmingen's manual.

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#### Radioligand binding assays

Cells may be screened for the presence of endogenous human receptor using radioligand binding or functional assays (described in detail in the following experimental description). Cells with either no or a low level of the endogenous human receptors disclosed herein present may be transfected with the human receptors.

Transfected cells from culture flasks are scraped into 5 ml of 20 mM Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates are centrifuged at 1000 rpm for 5 min. at 4°C, and the supernatant is centrifuged at 30,000 x g for 20 min. at 4°C. The pellet is suspended in binding buffer (50 mM Tris-HCl, 60 mM NaCl, 1 mM MgCl, 33μM EDTA, 33 μM EGTA at pH 7.4 supplemented with 0.2% BSA, 2 μg/ml aprotinin, and 20 μM bestatin). Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10% of the added radioligand, are added to 96-well polpropylene microtiter plates containing <sup>3</sup>H-labeled compound, unlabeled compounds, and binding buffer to a final volume of 250 μl. In equilibrium saturation binding assays membrane preparations are incubated in the presence of increasing concentrations of [<sup>3</sup>H]-labeled compound. The binding affinities of the different compounds are determined in equilibrium competition binding assays, using [<sup>125</sup>I]-labeled compound in the presence of ten to twelve different concentrations of the displacing ligands. Competition assay: 50pM radioligand, 10 - 12 points. Binding reaction mixtures are incubated for 2 hr at 25°C, and the reaction stopped by filtration through a double layer of GF filters treated with 0.1% polyethyleneimine, using a cell harvester. Wash buffer: 50mM Tris-HCl, 0.1% BSA. Radioactivity may be measured by scintillation counting and

data are analyzed by a computerized non-linear regression program. Non-specific binding is defined as the amount of radioactivity remaining after incubation of membrane protein in the presence of 1 $\mu$ M final concentration unlabeled. Protein concentration may be measured by the Bradford method using Bio-Rad Reagent, with bovine serum albumin as a standard.

#### Functional assays

Cells may be screened for the presence of endogenous mammalian receptor using radioligand binding or functional assays (described in detail in the above or following experimental description, respectively). Cells with no or a low level of endogenous receptor present may be transfected with the mammalian receptor for use in the following functional assays.

A wide spectrum of assays can be employed to screen for the presence of receptor ligands. These range from traditional measurements of phosphatidyl inositol, cAMP, Ca<sup>++</sup>, and K<sup>+</sup>, for example; to systems measuring these same second messengers but which have been modified or adapted to be higher throughput, more generic, and more sensitive; to cell based platforms reporting more general cellular events resulting from receptor activation such as metabolic changes, differentiation, and cell division/proliferation, for example; to high level organism assays which monitor complex physiological or behavioral changes thought to be involved with receptor activation including cardiovascular, analgesic, orexigenic, anxiolytic, and sedation effects, for example.

#### Cyclic AMP (cAMP) formation assay

The receptor-mediated inhibition of cyclic AMP (cAMP) formation may be assayed in transfected cells expressing the mammalian receptors. Cells are plated in 96-well plates and incubated in Dulbecco's phosphate buffered saline (PBS) supplemented with 10 mM HEPES, 5mM theophylline, 2  $\mu$ g/ml



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aprotinin, 0.5 mg/ml leupeptin, and 10  $\mu$ g/ml phosphoramidon for 20 min at 37°C, in 5% CO<sub>2</sub>. Test compounds are added and incubated for an additional 10 min at 37°C. The medium is then aspirated and the reaction stopped by the addition of 100 mM HCl. The plates are stored at 4°C for 15 min, and the cAMP content in the stopping solution measured by radioimmunoassay. Radioactivity may be quantified using a gamma counter equipped with data reduction software.

10 Arachidonic acid release assay

Cells stably transfected with the mammalian receptor are seeded into 96 well plates and grown for 3 days in HAM's F-12 with supplements. <sup>3</sup>H-arachidonic acid (specific activity = 0.75  $\mu$ Ci/ml) is delivered as a 100  $\mu$ L aliquot to each well and samples were incubated at 37° C, 5% CO<sub>2</sub> for 18 hours. The labeled cells are washed three times with 200  $\mu$ L HAM's F-12. The wells are then filled with medium (200  $\mu$ L) and the assay is initiated with the addition of peptides or buffer (22  $\mu$ L). Cells are incubated for 30 min at 37°C, 5% CO<sub>2</sub>. Supernatants are transferred to a microtiter plate and evaporated to dryness at 75°C in a vacuum oven. Samples are then dissolved and resuspended in 25  $\mu$ L distilled water. Scintillant (300  $\mu$ L) is added to each well and samples are counted for <sup>3</sup>H in a Trilux plate reader. Data are analyzed using nonlinear regression and statistical techniques available in the GraphPAD Prism package (San Diego, CA).

Intracellular calcium mobilization assay

The intracellular free calcium concentration may be measured by microspectrofluorimetry using the fluorescent indicator dye Fura-2/AM (Bush et al, 1991). Stably transfected cells are seeded onto a 35 mm culture dish containing a glass coverslip insert. Cells are washed with HBS and loaded with 100  $\mu$ L of Fura-2/AM (10  $\mu$ M) for 20 to 40 min. After washing with HBS to remove the Fura-2/AM solution, cells are equilibrated in HBS for 10 to 20 min. Cells are then visualized under the 40X objective of a Leitz Fluovert FS microscope and fluorescence emission is determined at 510 nm

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with excitation wavelengths alternating between 340 nM and 380 nM. Raw fluorescence data are converted to calcium concentrations using standard calcium concentration curves and software analysis techniques.

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Alternatively, cells expressing the mammalian receptor DNA are plated in 96-well plates and grown to confluence. Cells are incubated with a cell permeant fluorescent calcium indicator such as, but not restricted to, Fluo-3/AM. After washing with HBS to remove the Fluo-3/AM solution, cells are equilibrated for 20 min. The fluorescence emission due to intracellular calcium mobilization elicited by agonists of the expressed mammalian receptor, is determined with a fluorescence imaging plate reader (FLIPR, Molecular Devices Corporation, Sunnyvale, CA).

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#### Phosphoinositide metabolism assay

Cells stably expressing the mammalian receptor cDNA are plated in 96-well plates and grown to confluence. The day before the assay the growth medium is changed to 100  $\mu$ l of medium containing 1% serum and 0.5  $\mu$ Ci [ $^3$ H]myo-inositol, and the plates are incubated overnight in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> at 37°C). Alternatively, arachidonic acid release may be measured if [ $^3$ H]arachidonic acid is substituted for the [ $^3$ H]myo-inositol. Immediately before the assay, the medium is removed and replaced by 200  $\mu$ L of PBS containing 10 mM LiCl, and the cells are equilibrated with the new medium for 20 min. During this interval cells are also equilibrated with the antagonist, added as a 10  $\mu$ L aliquot of a 20-fold concentrated solution in PBS. The [ $^3$ H]inositol-phosphates accumulation from inositol phospholipid metabolism may be started by adding 10  $\mu$ L of a solution containing the agonist. To the first well 10  $\mu$ L may be added to measure basal accumulation, and 11 different concentrations of agonist are assayed in the following 11 wells of each plate row. All assays are performed in duplicate by repeating the same additions in two consecutive plate rows. The plates

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are incubated in a CO<sub>2</sub> incubator for 1 hr. The reaction may be terminated by adding 15  $\mu$ L of 50% v/v trichloroacetic acid (TCA), followed by a 40 min. incubation at 4 °C. After neutralizing TCA with 40  $\mu$ L of 1 M Tris, the content of the wells may be transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates are prepared adding 200  $\mu$ L of Dowex AG1-X8 suspension (50% v/v, water: resin) to each well. The filter plates are placed on a vacuum manifold to wash or elute the resin bed. Each well is washed 2 times with 200  $\mu$ L of water, followed by 2 x 200  $\mu$ L of 5 mM sodium tetraborate/60 mM ammonium formate. The [<sup>3</sup>H]IPs are eluted into empty 96-well plates with 200  $\mu$ L of 1.2 M ammonium formate/0.1 formic acid. The content of the wells is added to 3 ml of scintillation cocktail, and the radioactivity is determined by liquid scintillation counting.

#### GTP $\gamma$ S functional assay

Membranes from cells transfected with the mammalian receptors are suspended in assay buffer (50 mM Tris, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 7.4) supplemented with 0.1% BSA, 0.1% bacitracin and 10  $\mu$ M GDP. Membranes are incubated on ice for 20 minutes, transferred to a 96-well Millipore microtiter GF/C filter plate and mixed with GTP $\gamma$ S<sup>35</sup> (e.g., 250,000 cpm/sample, specific activity ~1000 Ci/mmol) plus or minus GTP $\gamma$ S (final concentration = 100  $\mu$ M). Final membrane protein concentration = 90  $\mu$ g/ml. Samples are incubated in the presence or absence of porcine galanin (final concentration = 1  $\mu$ M) for 30 min. at room temperature, then filtered on a Millipore vacuum manifold and washed three times with cold assay buffer. Samples collected in the filter plate are treated with scintillant and counted for <sup>35</sup>S in a Trilux (Wallac) liquid scintillation counter. It is expected that optimal results are obtained when the mammalian receptor membrane preparation is derived from an appropriately engineered heterologous expression system, i.e., an expression system resulting in high levels of expression of the mammalian receptor and/or expressing

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G-proteins having high turnover rates (for the exchange of GDP for GTP). GTPγS assays are well-known in the art, and it is expected that variations on the method described above, such as are described by e.g., Tian et al. (1994) or Lazareno and Birdsall (1993), may be used by one of ordinary skill in the art.

#### MAP kinase assay

MAP kinase (mitogen activated kinase) may be monitored to evaluate receptor activation. MAP kinase is activated by multiple pathways in the cell. A primary mode of activation involves the ras/raf/MEK/MAP kinase pathway. Growth factor (tyrosine kinase) receptors feed into this pathway via SHC/Grb-2/SOS/ras. Gi coupled receptors are also known to activate ras and subsequently produce an activation of MAP kinase. Receptors that activate phospholipase C (Gq and G11) produce diacylglycerol (DAG) as a consequence of phosphatidyl inositol hydrolysis. DAG activates protein kinase C which in turn phosphorylates MAP kinase.

MAP kinase activation can be detected by several approaches. One approach is based on an evaluation of the phosphorylation state, either unphosphorylated (inactive) or phosphorylated (active). The phosphorylated protein has a slower mobility in SDS-PAGE and can therefore be compared with the unstimulated protein using Western blotting. Alternatively, antibodies specific for the phosphorylated protein are available (New England Biolabs) which can be used to detect an increase in the phosphorylated kinase. In either method, cells are stimulated with the mitogen and then extracted with Laemmli buffer. The soluble fraction is applied to an SDS-PAGE gel and proteins are transferred electrophoretically to nitrocellulose or Immobilon. Immunoreactive bands are detected by standard Western blotting technique. Visible or chemiluminescent signals are recorded on film and may be quantified by densitometry.

Another approach is based on evaluation of the MAP kinase

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activity via a phosphorylation assay. Cells are stimulated with the mitogen and a soluble extract is prepared. The extract is incubated at 30°C for 10 min with gamma-<sup>32</sup>P-ATP, an ATP regenerating system, and a specific substrate for MAP kinase such as phosphorylated heat and acid stable protein regulated by insulin, or PHAS-I. The reaction is terminated by the addition of H<sub>3</sub>PO<sub>4</sub> and samples are transferred to ice. An aliquot is spotted onto Whatman P81 chromatography paper, which retains the phosphorylated protein. The chromatography paper is washed and counted for <sup>32</sup>P in a liquid scintillation counter. Alternatively, the cell extract is incubated with gamma-<sup>32</sup>P-ATP, an ATP regenerating system, and biotinylated myelin basic protein bound by streptavidin to a filter support. The myelin basic protein is a substrate for activated MAP kinase. The phosphorylation reaction is carried out for 10 min at 30°C. The extract can then be aspirated through the filter, which retains the phosphorylated myelin basic protein. The filter is washed and counted for <sup>32</sup>P by liquid scintillation counting.

#### Cell proliferation assay

Receptor activation of a G protein coupled receptor may lead to a mitogenic or proliferative response which can be monitored via <sup>3</sup>H-thymidine uptake. When cultured cells are incubated with <sup>3</sup>H-thymidine, the thymidine translocates into the nuclei where it is phosphorylated to thymidine triphosphate. The nucleotide triphosphate is then incorporated into the cellular DNA at a rate that is proportional to the rate of cell growth. Typically, cells are grown in culture for 1-3 days. Cells are forced into quiescence by the removal of serum for 24 hrs. A mitogenic agent is then added to the media. 24 hrs later, the cells are incubated with <sup>3</sup>H-thymidine at specific activities ranging from 1 to 10 uCi/ml for 2-6 hrs. Harvesting procedures may involve trypsinization and trapping of cells by filtration over GF/C filters with or without a prior incubation in TCA to extract soluble thymidine. The filters are processed with scintillant and counted for <sup>3</sup>H by liquid

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scintillation counting. Alternatively, adherant cells are fixed in MeOH or TCA, washed in water, and solubilized in 0.05% deoxycholate/0.1 N NaOH. The soluble extract is transferred to scintillation vials and counted for  $^3\text{H}$  by liquid scintillation counting.

#### Promiscuous second messenger assays

It is possible to coax receptors of different functional classes to signal through a pre-selected pathway through the use of promiscuous  $G_\alpha$  subunits. For example, by providing a cell based receptor assay system with an endogenously supplied promiscuous  $G_\alpha$  subunit such as  $G_{\alpha 16}$  or a chimeric  $G_\alpha$  subunit such as  $G_{\alpha zq}$ , a GPCR, which might normally prefer to couple through a specific signaling pathway (e.g.,  $G_g$ ,  $G_i$ ,  $G_q$ ,  $G_{12}$ , etc.), can be made to couple through the pathway defined by the promiscuous  $G_\alpha$  subunit and upon agonist activation produce the second messenger associated with that subunit's pathway. In the case of  $G_{\alpha 16}$  and/or  $G_{\alpha zq}$  this would involve activation of the  $G_q$  pathway and production of the second messenger phosphatidylinositol. Through the use of similar strategies and tools, it is possible to bias receptor signaling through pathways producing other second messengers such as  $\text{Ca}^{++}$ , cAMP, and  $\text{K}^+$  currents, for example.

#### Microphysiometric measurement of receptor mediated extracellular acidification rates

Because cellular metabolism is intricately involved in a broad range of cellular events (including receptor activation of multiple messenger pathways), the use of microphysiometric measurements of cell metabolism can in principle provide a generic assay of cellular activity arising from the activation of any receptor regardless of the specifics of the receptor's signaling pathway.

General guidelines for transient receptor expression, cell preparation and microphysiometric recording are described elsewhere (Salon, J.A. and Owicki, J.A., 1996). Receptors

and/or control vectors are transiently expressed in CHO-K1 cells, by liposome mediated transfection according to the manufacturers recommendations (LipofectAMINE, GibcoBRL, Gaithersburg, MD), and maintained in Ham's F-12 complete (10% serum). A total of 10 $\mu$ g of DNA is used to transfect each 75cm<sup>2</sup> flask which had been split 24 hours prior to the transfection and judged to be 70-80% confluent at the time of transfection. 24 hours post transfection, the cells are harvested and 3 x 10<sup>5</sup> cells seeded into microphysiometet capsules. Cells are allowed to attach to the capsule membrane for an additional 24 hours; during the last 16 hours, the cells are switched to serum-free F-12 complete to minimize ill-defined metabolic stimulation caused by assorted serum factors. On the day of the experiment the cell capsules are transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum (Molecular Devices Corporation, Sunnyvale, CA) containing 0.1% fatty acid free BSA), during which a baseline measurement of basal metabolic activity is established.

A standard recording protocol specifies a 100 $\mu$ l/min flow rate, with a 2 min total pump cycle which includes a 30 sec flow interruption during which the acidification rate measurement is taken. Ligand challenges involve a 1 min 20 sec exposure to the sample just prior to the first post challenge rate measurement being taken, followed by two additional pump cycles for a total of 5 min 20 sec sample exposure. Typically, drugs in a primary screen are presented to the cells at 10  $\mu$ M final concentration. Follow up experiments to examine dose-dependency of active compounds is then done by sequentially challenging the cells with a drug concentration range that exceeds the amount needed to generate responses ranging from threshold to maximal levels. Peptides included in the microphysiometric screen included rat NPFF (FLFQPQRF-NH<sub>2</sub>) (SEQ ID NO: 45) and rat A-18-F-amide (AGEGLSSPFWSLAAPQRF-NH<sub>2</sub>) (SEQ ID NO: 46). Ligand samples are then washed out and the acidification rates reported are

expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge.

Receptor/G protein co-transfection studies

5 A strategy for determining whether NPFF can couple preferentially to selected G proteins involves co-transfection of NPFF receptor cDNA into a host cell together with the cDNA for a G protein alpha sub-unit. Examples of G alpha sub-units include members of the G $\alpha$ i/G $\alpha$ o class (including G $\alpha$ t2 and G $\alpha$ z), the G $\alpha$ q class, the G $\alpha$ s class, and the G $\alpha$ 12/13 class. A typical procedure involves transient transfection into a host cell such as COS-7. Other host cells may be used. A key consideration is whether the cell has a downstream effector (a particular  
10 adenylate cyclase, phospholipase C, or channel isoform, for example) to support a functional response through the G protein under investigation. G protein beta gamma sub-units native to the cell are presumed to complete the G protein heterotrimer; otherwise specific beta and gamma sub-units  
15 may be co-transfected as well. Additionally, any individual or combination of alpha, beta, or gamma subunits may be co-transfected to optimize the functional signal mediated by the receptor.

25 The receptor/G alpha co-transfected cells are evaluated in a binding assay, in which case the radioligand binding may be enhanced by the presence of the optimal G protein coupling or in a functional assay designed to test the receptor/G protein hypothesis. In one example, the NPFF  
30 receptor may be hypothesized to inhibit cAMP accumulation through coupling with G alpha sub-units of the G $\alpha$ i/G $\alpha$ o class. Host cells co-transfected with the NPFF receptor and appropriate G alpha sub-unit cDNA are stimulated with forskolin +/- NPFF agonist, as described above in cAMP  
35 methods. Intracellular cAMP is extracted for analysis by radioimmunoassay. Other assays may be substituted for cAMP inhibition, including GTP $\gamma$ <sup>35</sup>S binding assays and inositol phosphate hydrolysis assays. Host cells transfected with



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NPFF minus G alpha or with G alpha minus NPFF would be tested simultaneously as negative controls. NPFF receptor expression in transfected cells may be confirmed in <sup>125</sup>I-NPFF protein binding studies using membranes from transfected cells. G alpha expression in transfected cells may be confirmed by Western blot analysis of membranes from transfected cells, using antibodies specific for the G protein of interest.

10 The efficiency of the transient transfection procedure is a critical factor for signal to noise in an inhibitory assay, much more so than in a stimulatory assay. If a positive signal present in all cells (such as forskolin-stimulated cAMP accumulation) is inhibited only in the fraction of  
15 cells successfully transfected with receptor and G alpha, the signal to noise ratio will be poor. One method for improving the signal to noise ratio is to create a stably transfected cell line in which 100% of the cells express both the receptor and the G alpha subunit. Another method  
20 involves transient co-transfection with a third cDNA for a G protein-coupled receptor which positively regulates the signal which is to be inhibited. If the co-transfected cells simultaneously express the stimulatory receptor, the inhibitory receptor, and a requisite G protein for the  
25 inhibitory receptor, then a positive signal may be elevated selectively in transfected cells using a receptor-specific agonist. An example involves co-transfection of COS-7 cells with 5-HT4, NPFF1, and a G alpha sub-unit. Transfected cells are stimulated with a 5-HT4 agonist +/- NPFF1 protein.  
30 Cyclic AMP is expected to be elevated only in the cells also expressing NPFF1 and the G alpha subunit of interest, and a NPFF-dependent inhibition may be measured with an improved signal to noise ratio.

35 It is to be understood that the cell lines described herein are merely illustrative of the methods used to evaluate the binding and function of the mammalian receptors of the present invention, and that other suitable cells may be used

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in the assays described herein.

### Electrophysiology

#### Methods for recording currents in *Xenopus* oocytes

5 Oocytes were harvested from *Xenopus laevis* and injected with mRNA transcripts as previously described (Quick and Lester, 1994; Smith et al., 1997). NPFF receptors and  $G\alpha_{q/2}$  chimera synthetic RNA transcripts were synthesized using the T7 polymerase ("Message Machine," Ambion) from linearized  
10 plasmids or PCR products containing the complete coding region of the genes. Oocytes were injected with 10 ng NPFF receptors synthetic RNA and incubated for 3-8 days at 17 degrees. Three to eight hours prior to recording, oocytes were injected with 500 pg  $G\alpha_{q/2}$  mRNA in order to observe  
15 coupling to  $Ca^{++}$  activated Cl currents. Dual electrode voltage clamp (Axon Instruments Inc.) was performed using 3 M KCl-filled glass microelectrodes having resistances of 1-2 Mohm. Unless otherwise specified, oocytes were voltage clamped at a holding potential of -80 mV. During  
20 recordings, oocytes were bathed in continuously flowing (1-3 ml/min) medium containing 96 mM NaCl, 2 mM KCl, 1.8 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , and 5 mM HEPES, pH 7.5 (ND96). Drugs were applied either by local perfusion from a 10 ml glass capillary tube fixed at a distance of 0.5 mm from the  
25 oocyte, or by switching from a series of gravity fed perfusion lines.

Other oocytes may be injected with a mixture of receptor mRNAs and synthetic mRNA encoding the genes for  
30 G-protein-activated inward rectifiers (GIRK1 and GIRK4, U.S. Patent Nos. 5,734,021 and 5,728,535). Genes encoding G-protein inwardly rectifying  $K^+$  (GIRK) channels 1 and 4 (GIRK1 and GIRK4) may be obtained by PCR using the published sequences (Kubo et al., 1993; Dascal et al., 1993;  
35 Krapivinsky et al., 1995 and 1995b) to derive appropriate 5' and 3' primers. Human heart cDNA may be used as template together with appropriate primers.

Heterologous expression of GPCRs in Xenopus oocytes has been widely used to determine the identity of signaling pathways activated by agonist stimulation (Gundersen et al., 1983; Takahashi et al., 1987). Activation of the phospholipase C (PLC) pathway is assayed by applying test compound in ND96 solution to oocytes previously injected with mRNA for the mammalian receptor and observing inward currents at a holding potential of -80 mV. The appearance of currents that reverse at -25 mV and display other properties of the  $\text{Ca}^{++}$ -activated  $\text{Cl}^-$  (chloride) channel is indicative of mammalian receptor-activation of PLC and release of  $\text{IP}_3$  and intracellular  $\text{Ca}^{++}$ . Such activity is exhibited by GPCRs that couple to  $\text{G}_q$ .

Measurement of inwardly rectifying  $\text{K}^+$  (potassium) channel (GIRK) activity may be monitored in oocytes that have been co-injected with mRNAs encoding the mammalian receptor, GIRK1, and GIRK4. The two GIRK gene products co-assemble to form a G-protein activated potassium channel known to be activated (i.e., stimulated) by a number of GPCRs that couple to  $\text{G}_i$  or  $\text{G}_o$  (Kubo et al., 1993; Dascal et al., 1993). Oocytes expressing the mammalian receptor plus the two GIRK subunits are tested for test compound responsivity by measuring  $\text{K}^+$  currents in elevated  $\text{K}^+$  solution containing 49 mM  $\text{K}^+$ . Activation of inwardly rectifying currents that are sensitive to 300  $\mu\text{M}$   $\text{Ba}^{++}$  signifies the mammalian receptor coupling to a  $\text{G}_i$  or  $\text{G}_o$  pathway in the oocytes.

#### Localization of mRNA coding for rat NPFF1 receptors

Development of probes for NPFF1: To facilitate the production of radiolabeled, antisense RNA probes a fragment of the gene encoding rat NPFF1 was subcloned into a plasmid vector containing RNA polymerase promotor sites. The full length cDNA encoding the rat NPFF1 was digested with Sph I (nucleotides 766-1111), and this 345 nucleotide fragment was cloned into the Sph I site of pGEM 3z, containing both  $\text{sp6}$  and T7 RNA polymerase promotor sites. The construct was sequenced to confirm sequence identity and orientation. To

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synthesize antisense strands of RNA, this construct was linearized with Hind III and T7 RNA polymerase was used to incorporate radiolabeled nucleotide as described below.

5 A probe coding for the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, a constitutively expressed protein, was used concurrently. GAPDH is expressed at a relatively constant level in most tissue and its detection is used to compare expression levels of the rat NPFF1  
10 receptors genes in different regions.

**Synthesis of probes:** NPFF1 and GAPDH cDNA sequences preceded by phage polymerase promoter sequences were used to synthesize radiolabeled riboprobes. Conditions for the  
15 synthesis of riboprobes were: 0.25-1.0  $\mu$ g linearized DNA plasmid template, 1.5  $\mu$ l of ATP, GTP, UTP (10 mM each), 3  $\mu$ l dithiothreitol (0.1M), 30 units RNasin RNase inhibitor, 0.5-1.0  $\mu$ l (15-20 units/ $\mu$ l) RNA polymerase, 7.0  $\mu$ l transcription buffer (Promega Corp.), and 12.5  $\mu$ l  $\alpha^{32}$ P-CTP  
20 (specific activity 3,000Ci/mmol). 0.1 mM CTP (0.02-1.0  $\mu$ l) was added to the reactions, and the volumes were adjusted to 35  $\mu$ l with DEPC-treated water. Labeling reactions were incubated at 37°C for 60 minutes, after which 3 units of RQ1 RNase-free DNase (Promega Corp.) were added to digest the  
25 template. Riboprobes were separated from unincorporated nucleotides using Microspin S-300 columns (Pharmacia Biotech). TCA precipitation and liquid scintillation spectrometry were used to measure the amount of label incorporated into the probe. A fraction of all riboprobes  
30 synthesized was size-fractionated on 0.25 mm thick 7M urea, 4.5% acrylamide sequencing gels. These gels were apposed to screens and the autoradiograph scanned using a phosphorimager (Molecular Dynamics) to confirm that the probes synthesized were full-length and not degraded.

35

**Solution hybridization/ribonuclease protection assay (RPA):** For solution hybridization 2.0  $\mu$ g of mRNA isolated from tissues were used. Negative controls consisted of 30  $\mu$ g

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transfer RNA (tRNA) or no tissue blanks. All mRNA samples were placed in 1.5 ml microfuge tubes and vacuum dried. Hybridization buffer (40  $\mu$ l of 400 mM NaCl, 20 mM Tris, pH 6.4, 2 mM EDTA, in 80% formamide) containing 0.25-2.0 E<sup>6</sup> counts of each probe were added to each tube. Samples were heated at 95°C for 15 min, after which the temperature was lowered to 55°C for hybridization.

After hybridization for 14-18 hr, the RNA/probe mixtures were digested with RNase A (Sigma) and RNase T1 (Life Technologies). A mixture of 2.0  $\mu$ g RNase A and 1000 units of RNase T1 in a buffer containing 330 mM NaCl, 10 mM Tris (pH 8.0) and 5 mM EDTA (400  $\mu$ l) was added to each sample and incubated for 90 min at room temperature. After digestion with RNases, 20  $\mu$ l of 10% SDS and 50  $\mu$ g proteinase K were added to each tube and incubated at 37°C for 15 min. Samples were extracted with phenol/chloroform:isoamyl alcohol and precipitated in 2 volumes of ethanol for 1 hr at -70°C. Pellet Paint (Novagen) was added to each tube (2.0  $\mu$ g) as a carrier to facilitate precipitation. Following precipitation, samples were centrifuged, washed with cold 70% ethanol, and vacuum dried. Samples were dissolved in formamide loading buffer and size-fractionated on a urea/acrylamide sequencing gel (7.0 M urea, 4.5% acrylamide in Tris-borate-EDTA). Gels were dried and apposed to storage phosphor screens and scanned using a phosphorimager (Molecular Dynamics, Sunnydale, CA).

#### RT-PCR

For the detection of low levels of RNA encoding rat NPFF1, RT-PCR was carried out on mRNA extracted from rat tissue. Reverse transcription and PCR reactions were carried out in 50  $\mu$ l volumes using EzrTth DNA polymerase (Perkin Elmer). Primers with the following sequences were used:

RA Rsnorf2/NPFF F1:

CTCCTACTACCAACACTCCTCTCC (SEQ ID NO: 39)

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RA RSNORF2/NPFF1 B1:

ACGGGTTACGAGCATCCAG (SEQ ID NO: 40)

5 These primers will amplify 490 base pair fragment from  
nucleotide 574 to 1064.

Each reaction contained 0.2  $\mu$ g mRNA and 0.3  $\mu$ M of each  
primer. Concentrations of reagents in each reaction were:  
300  $\mu$ M each of dGTP, dATP, dCTP, dTTP; 2.5mM Mn(OAc)<sub>2</sub>; 50mM  
10 Bicine; 115 mM K acetate, 8% glycerol and 5 units EzrTth DNA  
polymerase. All reagents for PCR (except mRNA and  
oligonucleotide primers) were obtained from Perkin Elmer.  
Reactions were carried out under the following conditions:  
65°C, 60 min; 94°C, 2 min; (94°C, 1 min; 65°C, 1 min) 35  
15 cycles, 72°C, 10 min. PCR reactions were size fractionated  
by agarose gel electrophoresis using 10% polyacrylamide. DNA  
was stained with SYBR Green I (Molecular Probes, Eugene, OR)  
and scanned on a Molecular Dynamics (Sunnyvale, CA) Storm  
860 in blue fluorescence mode at 450 nm.

20 Positive controls for PCR reactions consisted of  
amplification of the target sequence from a plasmid  
construct, as well as reverse transcribing and amplifying a  
known sequence. Negative controls consisted of mRNA blanks  
25 as well as primer blanks. To confirm that the mRNA was not  
contaminated with genomic RNA, samples were digested with  
RNases before reverse transcription. Integrity of RNA was  
assessed by amplification of mRNA coding for GAPDH.

30 Localization of mRNA coding for NPFF-like receptors  
(hNPFF2) using RT-PCR

For the detection of low levels of RNA encoding hNPFF2  
RT-PCR was carried out on mRNA extracted from tissue.  
Reverse transcription and PCR reactions were carried out in  
35 50  $\mu$ l volumes using EzrTh DNA polymerase (Perkin Elmer).  
Primers with the following sequences were used:

JB 249: 5'-GATCAGTGGATTGGTCCAGGGAATATC-3' (SEQ ID NO: 41)

JB 250: 5'-CCAGGTAGATGTTGGCAAACAGCAC-3' (SEQ ID NO: 42)

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These primers will amplify a 332 base pair fragment from TMIII to TMV.

5 Each reaction contained 0.1 ug mRNA and 0.3uM of each primer. Concentrations of reagents in each reaction were 300uM each of dGTP, dATP, dCTP, dTTP, 2.5mM Mn(OAc)<sub>2</sub>, 50mM Bicine, 115 mM potassium acetate, 8% glycerol and 5 units EzrTth DNA polymerase. All reagents for PCR (except mRNA and oligonucleotide primers) were obtained from Perkin  
10 Elmer. Reactions were carried out under the following conditions: 65°C 60 min., 94°C 2 min, (94°C, 1 min, 85°C 1 min) 35 cycles, 72°C 10 min. PCR reactions were size fractionated by gel electrophoresis using 10% polyacrylamide. DNA was stained with SYBR Green I  
15 (Molecular Probes, Eugene OR) and scanned on a Molecular Dynamics (Sunnyvale, CA) Strom 860 in blue fluorescence mode at 450 nm.

20 Positive controls for PCR reactions consisted of amplification of the target sequence from a plasmid construct, as well as reverse transcribing and amplifying a known sequence. Negative controls consisted of mRNA blanks and primer blanks. To confirm that the mRNA was not contaminated with genomic DNA, samples were digested with  
25 RNases before reverse transcription. Integrity of RNA was assessed by amplification of mRNA coding for GAPDH.

#### Localization of mRNA coding for human and rat NPFF Receptors

#### 30 Materials and Methods

Quantitative RT-PCR using a fluorogenic probe with real time detection: Quantitative RT-PCR using fluorogenic probes and a panel of mRNA extracted from human and rat tissue was used  
35 to characterize the localization of NPFF rat and human RNA. This assay utilizes two oligonucleotides for conventional PCR amplification and a third specific oligonucleotide probe that is labeled with a reporter at the 5' end and a quencher

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at the 3' end of the oligonucleotide. In the instant invention, FAM (6-carboxyfluorescein) and JOE (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein) were the two reporters that were utilized and TAMRA (6-carboxy-4,7,2,7'-tetramethylrhodamine) was the quencher. As amplification progresses, the labeled oligonucleotide probe hybridizes to the gene sequence between the two oligonucleotides used for amplification. The nuclease activity of Taq, or rTth thermostable DNA polymerases is utilized to cleave the labeled probe. This separates the quencher from the reporter and generates a fluorescent signal that is directly proportional to the amount of amplicon generated. This labeled probe confers a high degree of specificity. Non-specific amplification is not detected as the labeled probe does not hybridize. All experiments were conducted in a PE7700 Sequence Detection System (Perkin Elmer, Foster City CA).

Quantitative RT-PCR: For the detection of RNA encoding NPFF receptors, quantitative RT-PCR was performed on mRNA extracted from tissue. Reverse transcription and PCR reactions were carried out in 50 ul volumes using rTth DNA polymerase (Perkin Elmer). Primers with the following sequences were used:

NPFF1 human:

Forward primer:

NPFF1h-913F

5'-CTGGTCACCGTCTACGCCTT-3' (SEQ ID NO: 60)

Reverse primer:

NPFF1h-1016R

5'-CCGCGGCGGAAGTTCT-3' (SEQ ID NO: 61)

Fluorogenic oligonucleotide probe:

NPFF1h-962T

5'-(6-FAM)-ACAGCAGCGCCAACCCCATCAT-(TAMRA)3' (SEQ ID NO: 62)



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**NPFF2 human:**

Forward primer:

NPFF2h-828F

5 5'-CCTGATTGTGGCCCTGCT-3' (SEQ ID NO: 63)

Reverse primer:

NPFF2h-916R

10 5'-CATTTGGAGAAAGGTCAGCGTAG-3' (SEQ ID NO: 64)

Fluorogenic oligonucleotide probe:

NPFF2h-855T

5'-(6-FAM)-CTCATGGCTGCCCCCTGTGGACTCAAT-(TAMRA)3' (SEQ ID NO: 65)15 **NPFF1 rat**

Forward primer:

NPFF1r-412F

20 5'-GCTGTGGAAAGGTTCCGCT-3' (SEQ ID NO: 66)

Reverse primer:

NPFF1r-474R

5'-CGCCTTCCGAAGGGTCA-3' (SEQ ID NO: 67)

25 Fluorogenic oligonucleotide probe:

NPFF1r-433T

5'-(6-FAM)-ATCGTGCACCCTTTCCGCGAGAA-(TAMRA)3' (SEQ ID NO: 68)30 **NPFF2 rat**

Forward primer:

NPFF2r deg-690F

5'-GAGGATCTACACCACCGTGCTATT-3' (SEQ ID NO: 69)

35 Reverse primer:

NPFF2r deg-776R

5'-GAAGCCCCAATCCTTGCATAC-3' (SEQ ID NO: 70)

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Fluorogenic oligonucleotide probe:

NPFF2r-722T

5'(6-FAM)-TCTACCTGGCTCCACTCTCCCTCATTGTT-(TAMRA)3' (SEQ ID NO: 71)

5

Using these primer pairs, amplicon length is 103 bp for human NPFF1, 88 bp for human NPFF2, 62 bp for rat NPFF1, and 86 bp for rat NPFF2. Each human RT-PCR reaction contained 50 ng mRNA and each rat RT-PCR reaction contained 100 ng total RNA. Oligonucleotide concentrations were: 500 nM of forward and reverse primers, and 200 nM of fluorogenic probe. Concentrations of reagents in each reaction were: 300  $\mu$ M each of dGTP; dATP; dCTP; 600  $\mu$ M UTP; 3.0mM Mn(OAc)<sub>2</sub>; 50 mM Bicine; 115 mM potassium acetate, 8% glycerol, 5 units rTth DNA polymerase, and 0.5 units of uracil N-glycosylase. Buffer for RT-PCR reactions also contained a fluor used as a passive reference (ROX: Perkin Elmer proprietary passive reference I). All reagents for RT-PCR (except mRNA and oligonucleotide primers) were obtained from Perkin Elmer (Foster City, CA). Reactions were carried using the following thermal cycler profile: 50°C 2 min., 60°C 30 min., 95°C 5 min., followed by 40 cycles of: 94°C, 20 sec., 62°C 1 min.

Positive controls for PCR reactions consisted of amplification of the target sequence from a plasmid construct when available. Standard curves for quantitation of human and rat NPFF1 and NPFF2 were constructed using RNA extracted from whole brain. Negative controls consisted of mRNA blanks, as well as primer and mRNA blanks. To confirm that the mRNA was not contaminated with genomic DNA, PCR reactions were carried out without reverse transcription using Taq DNA polymerase. Integrity of RNA was assessed by amplification of RNA coding for cyclophilin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following reverse transcription and PCR amplification, data was analyzed using Perkin Elmer sequence detection software. The fluorescent signal from each well was normalized using

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an internal passive reference, and data was fitted a standard curve to obtain relative quantities of NPFF mRNA expression.

5

Receptor Autoradiographic Experiments Localizing NPFF  
Receptor Subtypes in the Rat CNS

Animals

10 Male Sprague-Dawley rats (Charles Rivers, Rochester, NY)  
were euthanized using CO<sub>2</sub>, decapitated, and their brains and  
peripheral tissues were immediately removed and rapidly  
frozen on crushed dry ice. Coronal sections of brain  
15 tissues were cut at 20µm using a cryostat, thaw-mounted onto  
gelatin-coated slides then stored at -20°C until binding  
assay.

Materials

20 [<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe)Phe<sup>3</sup>]NPFF (specific activity 2200 Ci/mmol  
was synthesized by iodination with chloramine-T from NEN  
(Boston, MA). BIBP 3226 was from RBI (Natick, MA). Frog  
pancreatic polypeptide (frog PP) (Rana Temporaria) was from  
Peninsula (Belmont, CA), and Neuropeptide FF (NPFF) was from  
25 (Bachem, King of Prussia, PA).

In vitro autoradiography

Tissue sections were allowed to equilibrate to room  
temperature for one hour. Sections were then incubated at  
30 25°C for 2 hours in 50 mM Tris-HCl buffer, pH 7.4, containing  
1 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1% Bovine Serum Albumin (Boehringer  
Mannheim, Indianapolis, IN) and 0.05 nM  
[<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe)Phe<sup>3</sup>]NPFF. Adjacent sections were  
incubated in the presence of 300 nM BIBP 3226 to selectively  
35 displace binding to NPFF1 or 300 nM frog PP to selectively  
displace binding to NPFF2. Nonspecific binding was  
determined by including 1 µM unlabeled NPFF in the  
incubation buffer. Sections were then washed four times, 5

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minutes each, in 4°C 50 mM Tris-buffer pH 7.4 then rapidly  
dipped in ice-cold distilled water to remove salts. Tissues  
were then dried under a stream of cold air. The sections  
were subsequently apposed together with <sup>125</sup>I-plastic standard  
5 scales, to Kodak BIOMAX MS Scientific Imaging Film (Eastman  
Kodak Company, Rochester, NY) for three days at room  
temperature. Films were developed using a Kodak M35A X-OMAT  
Processor (Eastman Kodak Company, Rochester, NY). Specific  
[<sup>125</sup>I][D-Tyr<sup>1</sup>-(NMe)Phe<sup>3</sup>]NPFF binding to NPFF1 and NPFF2  
10 receptors was interpreted by observation of the remaining  
optical density on the autoradiogram in the various regions  
of rat brain in the presence of the appropriate displacers.

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Results and DiscussionCloning and SequencingrNPFF1 and hNPFF1

100 ng genomic DNA was subjected to MOPAC PCR with two  
5 degenerate primers designed based on the sixth and seventh  
transmembrane domains of over 180 receptors from the  
rhodopsin superfamily of G protein-coupled receptors. Two  
products from this reaction, MPR3-RGEN-31 and MPR3-RGEN-45  
were found to be identical clones of a novel DNA sequence  
10 not found in the Genbank databases (Genembl, STS, EST, GSS),  
which had 30-40% amino acid identity with the known  
receptors dopamine D2, orexin 1, GALR1, angiotensin 1B and  
SHT-2b. This novel clone was given the name SNORF2.

15 The full-length SNORF2 sequence was acquired by screening  
rat hypothalamic cDNA libraries by PCR using specific SNORF2  
oligonucleotide primers. Pools of the rat hypothalamic cDNA  
library "I" were screened by PCR with SNORF2-specific primers  
JAB208 and JAB209. This screen yielded a positive pool I36.  
20 Successive PCR screening of sub-pools of this pool followed  
by high stringency hybridization of isolated colonies from  
the positive sub-pool I36-17 with the SNORF2-specific  
oligonucleotide probe indicated that the isolated clone  
I36E-17-1B-1 contained at least a partial clone of SNORF2.  
25 Sequencing of I36E-17-1B-1 revealed that this insert  
contained the coding region from the TMIII-TMIV loop through  
the stop codon, including some 3' untranslated sequence.  
From this sequence, a new forward primer, JAB221, was  
designed in TMV. PCR screening of a second rat hypothalamic  
30 cDNA library "J" with primers JAB221 and JAB209, and  
subsequent colony hybridization with the JAB211 probe on a  
low complexity positive sub-pool resulted in the isolation  
of a SNORF2 clone J-13-16-A1. This clone contained the  
full-length coding sequence of SNORF2 (1296bp) with  
35 approximately 200 bp 5'untranslated sequence and 1.3 kb 3'  
untranslated sequence. The nucleotide sequence of SNORF2  
and its translated amino acid sequence are represented in  
Figures 1 and 2, respectively. As shown in Figure 1, SNORF2

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contains two potential initiating methionines upstream of TMI.

Hydrophobicity (Kyte-Doolittle) analysis of the amino acid  
5 sequence of the full-length clone indicates the presence of  
seven hydrophobic regions, which is consistent with the  
seven transmembrane domains of a G protein-coupled receptor.  
The seven expected transmembrane domains are mapped out in  
Figure 3. A comparison of nucleotide and peptide sequences  
10 of SNORF2 with sequences contained in the  
Genbank/EMBL/SwissProtPlus databases reveals that the amino  
acid sequence of this clone is most related to the orexin 1  
and 2 receptors (45% and 40% identity, respectively) as well  
as the neuropeptide Y receptors Y1, Y2 and Y4 (~30%  
15 identity). Further homology analysis of SNORF2 against the  
Synaptic Pharmaceutical Corporation in-house database  
revealed that SNORF2 has a very high degree of identity with  
a proprietary Synaptic Pharmaceutical Corporation human  
partial GPCR clone named PLC29b (85% nucleotide identity,  
20 93% amino acid identity). PLC29b was originally isolated  
from a human genomic library using oligonucleotide probes  
for NPY4, and includes part of the amino terminus and TMs I  
to IV. Partial nucleotide and amino acid sequence of PLC29b  
(human SNORF2) is represented in Figures 4 and 5,  
25 respectively. Based on sequence similarity, PLC29b appears  
to be a partial clone of the human homologue of SNORF2.  
Therefore, this human homolog of SNORF2 has been named  
hNPFF1. A GAP alignment demonstrating the high homology  
between these species homologues is represented in Figure 6.

30  
SNORF2 has several potential protein kinase C (PKC)  
phosphorylation motifs throughout its amino acid sequence:  
threonine 154 in the second intracellular loop, threonine  
263 and serine 264 in the third intracellular loop, and  
35 serine 363 in the intracellular carboxy-terminal tail. It  
also has four potential N-linked glycosylation sites at  
asparagines 10 and 18 in the amino-terminal tail and at  
asparagines 113 and 195 in the first and second

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extracellular loops, respectively.

### hNPFF2

5 In analyzing the sequence of rNPFF1 and its homology to  
other sequences in GenBank, a 532 bp EST with the accession  
number AA449919 was identified which had a high degree of  
identity to rNPFF1. The translation of this sequence  
indicated that it coded for the region between the first  
10 extracellular loop and the beginning of the sixth  
transmembrane domain of a G protein-coupled receptor (GPCR).  
Although AA449919 was documented as being similar to the  
Drosophila melanogaster NPY receptor (accession number  
P25931), it was found that the amino acid sequence encoded  
by this EST was much more similar to NPFF1. The predicted  
15 amino acid sequence of AA449919 and rNPFF1 are 50%  
identical, while the amino acid sequence of the Drosophila  
NPY receptor is only 31% identical to the translation of  
AA449919. Because of the high degree of identity between  
AA449919 and rNPFF1, AA449919 was given the name hNPFF2,  
20 representing a member of a novel family of NPFF receptors of  
which there is currently only one member, NPFF1.

The full length sequence of NPFF-like (hNPFF2) was acquired  
by 5'/3' RACE using human spleen cDNA as a template, as  
25 described above, demonstrating that the coding region of  
hNPFF-like (hNPFF2) is 1260 bp, coding for a protein of 420  
amino acids. Sequencing of clones from several independent  
PCR reactions using spleen, heart, and spinal cord cDNA as  
templates and subsequent alignment of these clones with  
30 Sequencher 3.0 was used to confirm the sequence of  
hNPFF-like (hNPFF2). The full-length nucleotide sequence of  
human NPFF2 is shown in Figure 7, and its translated amino  
acid sequence is shown in Figure 8. The seven putative  
transmembrane domains of hNPFF-like (hNPFF2) are defined in  
35 Figure 9.

Like the original EST AA449919, the amino acid sequence  
encoded by the full-length DNA sequence of hNPFF2 is most

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similar to rNPFF1 (48% identity), as shown in the GAP alignment between the two receptors in Figure 10. The next-best matches in SWPLUS to full-length hNPFF2 are the Drosophila NPYR (accession number P25931, 34% identity) and TLR2 (accession number P30975, 32% identity), human orexin 1 and 2 receptors (O43613, 31% and O43614, 29%, respectively) and human NPY1 and Y4 receptors (P25929, 31% and P50391, 32%, respectively). A Blast search of the EST database using the full-length nucleotide sequence of hNPFF2 revealed an EST (Accession number AA449920) that is identical to hNPFF2 from the end of TM7 through the stop codon. ESTs AA44919 and AA44920 are the same clone sequenced from 5' end or the 3' end, respectively.

hNPFF2 contains several potential N-linked glycosylation sites. The first three sites, asparagines 8, 20, and 31 are in the N-terminal extracellular domain. Another potential N-linked glycosylation site, at position 198, is in the second extracellular loop. This receptor also contains one potential PKC phosphorylation site at threonine 156 in the second intracellular loop, and two potential PKC phosphorylation sites in the third intracellular loop at threonine 254 and serine 266.

#### 25 hNPFF1

The sequence of hNPFF1 from the initiating methionine to TMIV was determined to be present in a partial clone, plc29b, found in a Synaptic Pharmaceutical Corporation in-house database. Additional sequence, including TMIV through the stop codon, was determined by sequencing a vector-anchored PCR product from a human cosmid library clone identified by hybridization with a <sup>32</sup>P-labeled probe (BB609) corresponding to the II/III loop of plc29b. Next, a human spinal cord library was screened by PCR using primers designed against the partial hNPFF1 sequence, BB729 and BB728. One positive pool, W4, was subdivided and a positive sub-pool was screened by colony hybridization with a <sup>32</sup>P-labeled probe from TMII of hNPFF1, BB676. Plasmid DNA



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was isolated for clone W4-18-4, renamed BO98, and DNA sequencing revealed that it was full-length but in the wrong orientation for expression in the expression vector pEXJ. To obtain a full-length hNPFF1 construct in the correct orientation, BO98 was amplified with BB757 and BB758, and the resulting product ligated into pcDNA3.1 and transformed into DH5 $\alpha$  cells. The sequence of one of these transformants was identical to the hNPFF1 sequence previously determined from the consensus of BO98 and the two cosmid clones. This human NPFF1 construct in pcDNA3.1 in the correct orientation was renamed BO102.

The hNPFF1 clone contains an open reading frame with 1293 nucleotides and predicts a protein of 430 amino acids (Figures 11 and 12). Seven transmembrane domains predicted by hydrophobicity analysis are indicated in Figure 13. The sequence of hNPFF1 was determined to be most similar to the rat NPFF1 (86% nucleotide identity, 87% amino acid identity) and human NPFF2 (56% nucleotide identity, 49% amino acid identity (Figure 14)). The human NPFF1 receptor also shares homology with human orexin<sub>1</sub> (53% nucleotide identity, 35% amino acid identity), human orexin<sub>2</sub> (43% nucleotide identity, 33% amino acid identity), human NPY<sub>2</sub> (47% nucleotide identity, 31% amino acid identity), human CCK<sub>A</sub> (46% nucleotide identity, 32% amino acid identity), and human CCK<sub>B</sub> (46% nucleotide identity, 26% amino acid identity).

#### Isolation of the Rat NPFF2 Homologue

A fragment of the rat homologue of NPFF2, from TMIV to TMVI, was amplified from rat genomic DNA, rat hypothalamic cDNA and rat spinal cord cDNA by reduced stringency PCR using oligonucleotide primers designed against the human NPFF2. Additional sequence was obtained by amplifying rat spinal cord cDNA under reduced stringency using PCR primers designed against the rat NPFF2 fragment along with primers corresponding to the NH<sub>2</sub>- and COOH-termini of the human NPFF2 receptor. This resulted in the identification of a rat NPFF2 fragment from TMI to TMVII.

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The remaining sequence of the rat NPFF2 receptor was acquired by screening a rat genomic phage library with an oligonucleotide probe corresponding to the second extracellular loop and TMV of rat NPFF2. Southern blot analysis of three isolated plaques with this same probe identified a 3.5 kb fragment which was subcloned and sequenced, revealing the COOH terminus and some 3'UT. A subsequent Southern blot analysis using an oligonucleotide probe corresponding to TMI of rat NPFF2 identified a 2.1 kb fragment which was subcloned and sequenced, revealing the NH<sub>2</sub> terminus and some 5'UT.

The full-length rat NPFF2 clone was amplified from rat spinal cord cDNA using a sense PCR primer corresponding to the 5'UT and an antisense primer corresponding to the 3'UT, and subcloned into pcDNA3.1. Sequencing of 5 independent PCR products revealed an open reading frame of 1251 bp that is predicted to encode a protein of 417 amino acids (Figures 22A-C and 23A-B, respectively). In addition, several potential allelic variations were identified and verified by sequencing additional genomic DNA PCR products. The allelic variations are at the following nucleotide positions (relative to Figures 22A-C): position 913 can be either G or A, position 949 can be either C or T, position 955 can be either C or T, and position 1151 can be either C or T. None of these variants alter the predicted amino acid sequence. One construct, whose nucleotide sequence is shown in Figures 22A-C was renamed pcDNA3.1-rNPFF2-f.

Hydrophobicity (Kyte-Doolittle) analysis of the amino acid sequence of the full-length clone indicates the presence of seven hydrophobic regions, which is consistent with the seven transmembrane domains of a G protein-coupled receptor. The seven expected transmembrane domains are indicated in Figures 23A-B. A comparison of nucleotide and peptide sequences of rat SNORF33 with sequences contained in the Genbank, EMBL and SwissProtPlus databases reveals that the nucleotide sequence of this clone is 81% identical to an

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orphan GPCR NPGPR (GenBank accession number AF119815), and the amino acid sequence of this clone is most related to orexin-1 and orexin-2 (34% amino acid identities), NPY2 (32% amino acid identity) and GIR (31% amino acid identity). There were no sequences in the Genbank databases (Genembl, STS, EST, GSS, or SwissProt) that were identical to rat NPFF2. The rat and human NPFF2 receptors share 81% nucleotide and 78% amino acid identities (Figure 24). The rat NPFF2 and rat NPFF1 receptors share 55% nucleotide and 50% amino acid identities (Figure 25).

Rat NPFF2 has five potential N-linked glycosylation sites, at asparagines 8, 20 and 31 in the amino-terminal tail, at asparagine 198 in the second extracellular loop and at asparagine 324 in the seventh transmembrane domain. It also has three potential protein kinase C (PKC) phosphorylation motifs at threonine 156 in the second intracellular loop, and at threonine 254 and serine 265 in the third intracellular loop. NPFF2 also has two potential casein kinase II phosphorylation sites at threonine 102 in the second transmembrane domain and at serine 403 in the carboxy-terminal tail.

### Electrophysiology

#### NPFF1

Oocytes injected with both SNORF2 and chimeric  $G\alpha_{q/2}$  synthetic RNAs generated robust inward currents in response to NPFF and the related peptide A-18-F-amide at 1  $\mu$ M (Figures 15A,B). Control oocytes receiving only G-protein synthetic RNA were unresponsive to these peptides. Responses to NPFF were concentration-dependent with a threshold for activation of inward current at 30 nM. The C-terminal tetrapeptide PQRF-amide also elicited responses at a concentration of 10  $\mu$ M (Figure 15C). Analogs of NPFF containing a tyrosine residue at the N-terminus or internally including Y-8-F-amide, [tyr<sup>9</sup>]A-18-F-amide and Y-18-F-amide also displayed activity at 1  $\mu$ M. Unrelated neuropeptides and other neurotransmitters including melanin concentrating

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hormone, orexin B, PYY, 5-HT, nociceptin, galanin and CCK failed to activate oocytes injected with the SNORF2 synthetic RNA. The functional responsiveness to NPFF and related peptides strongly suggests that SNORF2 encodes a  
5 receptor for neuropeptide FF (NPFF); therefore SNORF2 was renamed NPFF1. Similarly, SNORF2-like was renamed NPFF-like.

Oocytes injected with NPFF1 and not the chimeric G-protein synthetic RNA failed to generate responses to NPFF. This  
10 observation supports the hypothesis that NPFF1 couples to G-proteins of the  $G\alpha_i/G\alpha_o/G\alpha_z$  class, and by virtue of the N-terminal portion of  $G\alpha_{q/z}$ , subsequently activates phospholipase C. In oocytes expressing both NPFF1 and  $G\alpha_{q/z}$ ,  
15  $Cl^-$  currents were abolished by prior injection of 10 mM EGTA, demonstrating the  $Ca^{++}$  dependence of these currents.

#### NPFF2

Oocytes injected with both the NPFF-like PCR product and  
20 chimeric  $G\alpha_{q/z}$  synthetic RNAs generated large inward currents in response to 1  $\mu M$  NPFF (Figure 16A). A-18-F-amide and PGRF-amide also at 1  $\mu M$  activated similar inward currents, although the magnitude of currents generated by PGRF-amide were smaller. No activity was observed using FMRF-amide at  
25 1  $\mu M$ . The unrelated neuropeptides orexin A, NPY, galanin, and neurokinin A at 1  $\mu M$  also failed to activate responses in oocytes injected with NPFF-like mRNA (Figure 16B). Oocytes injected with both the NPFF-like plasmid (BO89) and chimeric  $G\alpha_{q/z}$  synthetic RNAs also produced robust currents  
30 in response to NPFF (Figure 16C). Based on these results, NPFF-like was renamed NPFF2. Oocytes injected with NPFF2 and not chimeric G-protein mRNA failed to generate responses to NPFF. This observation supports the hypothesis that NPFF2 couples to G-proteins of the  $G\alpha_i/G\alpha_o/G\alpha_z$  class, and by  
35 virtue of the N-terminal portion of  $G\alpha_{q/z}$ , subsequently activates phospholipase C.

#### Microphysiology

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CHO cells transiently expressing either NPFF1 alone or NPFF1 accompanied by the chimeric protein Gq/Gz produced robust increases in metabolism when exposed to either NPFF or the related peptide A-18-F-amide as evidenced by increased rates of extracellular acidification when measured by the microphysiometric technique (Figures 17A and 17B). Whereas control cells, not expressing NPFF1, produced no increase in acidification rates to either NPFF or A-18-F-amide. In all cases the NPFF1 mediated responses were dose-dependent. CHO cells transfected with NPFF1 alone produced an EC<sub>50</sub> value of 19.3 nM for NPFF while cells transfected NPFF1 and the chimeric Gz/Gq produced an EC<sub>50</sub> of 27.7 nM for NPFF. Challenges with A-18-F-amide were conducted only on cells that had been transfected with NPFF1 alone. These cells produced an EC<sub>50</sub> value of 150nM for A-18-F-amide. The functional responsiveness to NPFF and A-18-F-amide supports the notion that NPFF1 encodes a receptor for neuropeptide FF (NPFF).

#### Radioligand binding assays

Cos-7 cells transiently expressing the gene encoding the novel rat NPFF1 receptor were used for pharmacological evaluation. Membranes harvested from transiently transfected Cos-7 cells exhibited high affinity, saturable [<sup>125</sup>I]D-Tyr-NPFF ([D-Tyr<sup>1</sup>(NMe)Phe<sup>3</sup>]NPFF) binding. Nonlinear analysis of [<sup>125</sup>I]D-Tyr-NPFF saturation data yielded an equilibrium dissociation constant (K<sub>d</sub>) of 0.335 ± 0.045 nM and a binding site density (B<sub>max</sub>) of 180 ± 11 fmol/mg protein. Specific [<sup>125</sup>I]D-Tyr-NPFF binding was about 50% of total binding at a radioligand concentration equal to the K<sub>d</sub> value. Mock-transfected host cells did not display specific [<sup>125</sup>I]D-Tyr-NPFF binding.

To further assess the pharmacological identity of the newly isolated NPFF1 receptor gene, detailed binding properties of cloned NPFF1 receptor were determined from nonlinear analysis of competition of high affinity [<sup>125</sup>I]D-Tyr-NPFF binding. The rank order of affinity of compounds to

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displace specific [ $^{125}$ I]D-Tyr-NPFF binding is shown in Table 1.

5 The binding profile of rat NPFF1 was compared to that of rat spinal cord membranes. Interestingly some differences were observed in the pharmacological profile between the two preparations. (See \* Table 2). Notably, fPP did not displace the binding on the NPFF1 receptor up to 1  $\mu$ M whereas it displayed a high affinity at the rat spinal cord. 10 Furthermore, several compounds displayed significantly different affinities between NPFF1 receptor and the spinal cord membranes. These compounds are highlighted in Table 1 and are ([ $^{125}$ I]D-Tyr-NPFF, A18Famide, Y8Famide, [ $^9$ ]A18Famide, Dynorphin A 1-13, Neuropeptide F and Met-Enk-NH<sub>2</sub>. These 15 data indicate the presence of additional NPFF receptor subtypes on the rat spinal cord.

Additional pharmacological evaluation was done using 293 human embryonic kidney cells (HEK-293 cells) transiently 20 expressing the genes encoding the human NPFF1, NPFF2, and rat NPFF1 receptors, as well as Cos-7 cells expressing the rat NPFF2 receptors. Nonlinear analysis of [ $^{125}$ I]D-Tyr-NPFF saturation binding data yielded equilibrium dissociation constants ( $K_d$ ) of  $0.46 \pm 0.10$  and  $0.17 \pm 0.04$  nM for the 25 human NPFF1 and NPFF2, and of  $0.65 \pm 0.22$  and  $0.17 \pm 0.02$  nM for the rat NPFF1 and NPFF2 receptors, respectively. The binding affinities ( $pK_i$ ) of various NPFF-related peptides were derived from competition binding assay using [ $^{125}$ I]D-Tyr-NPFF as a ligand. In agreement with the data shown 30 in Table 1, fPP showed 31- and 77-fold greater affinity for the rat and human NPFF2 receptors, respectively, when compared to the NPFF1 receptors (see Table 3). The other peptides studied showed overall similar binding affinities for both the rat and human NPFF1 and NPFF2 receptors. NPFF 35 receptors displayed high affinity for FMRF amide and lower binding affinity for the D-Met analog, suggesting the existence of stereoselectivity for this peptide.

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The ability of NPFF1 receptors to functionally couple to PI was tested using intact Cos-7 cells transiently expressing NPFF1. Full dose-response curves were determined for NPFF-mediated total IP release (Figure 18A). NPFF stimulated total IP release with an EC50 of 23 nM and an Emax of approximately 200% basal. This weak stimulation was most probably mediated by NPFF1 coupling to a Gi/Go G-protein via  $\beta\gamma$ -induced PI turnover, since the response was abolished by pretreatment with pertussis toxin but not cholera toxin. In contrast, a robust stimulation of total IP release was observed following NPFF in Cos-7 cells transfected with both the NPFF1 receptor and the Gq/Gz chimera (Figure 18B). NPFF stimulated total IP release with an EC50 of 2.95 nM, and an Emax of approximately 1500% basal. As anticipated, neither PTX nor CTX attenuated this response. Similar to what was observed in oocytes, this suggests a coupling in Cos-7 cells to G-proteins of the G $\alpha$ i/G $\alpha$ o/G $\alpha$ z class.

The coupling of human NPFF1 and NPFF2 receptors to the activation of intracellular second messenger pathways was studied further in COS-7 cells co-transfected with the Gq/Gz chimera. In such cells, NPFF elicited an increase in intracellular calcium when either the human NPFF1 or NPFF2 were transfected, and no response was observed in cells that were only transfected with the Gq/Gz chimera. As shown in Table 4, PQRF amide was a full agonist in cells expressing either the NPFF1 or NPFF2 receptors. However, only cells expressing the human NPFF2 responded with an intracellular calcium response to fPP, no response was observed in cells expressing the human NPFF1 receptor, suggesting that fPP is an NPFF2-selective agonist.

Table 1

pKi for cloned rat NPFF1 receptor binding in COS-7 cells

	COMPOUND	MEAN	SEM	n
	NPFF (F-8-Fa)	8.535	0.02	2
5	(D-Tyr <sup>1</sup> - (NMe) Phe <sup>3</sup> ) NPFF	8.549	0.13	4
	A18Fa	7.495	0.11	2
	PQRFa	8.182	0.03	2
	FMRFa	8.481	0.05	2
	YFa	8.382	0.22	2
10	[Y <sup>9</sup> ] A18Fa	7.558	0.12	2
	hPP	5.000	0.00	2
	fPP	5.500	0.35	2
	substance P	5.000	0.00	2
	Dynorphin A1-13	6.838	0.29	2
15	(3D) Y8Fa	8.623	0.44	4
	(2D) Y8Fa	8.330	0.15	4
	CCK8	5.000	0.00	2
	galanin	5.000	0.00	2
	dopamine	5.000	0.00	2
20	naloxone	5.000	0.00	2
	CGRP	5.000	0.00	2
	AF-1	6.634	0.13	2
	AF-2	7.023	0.41	2
	SchistFLRF	5.960	0.68	2
25	Met5-Arg6-Phe7-Enk-NH <sub>2</sub>	7.350	0.22	4
	Met5-Arg6-Phe7-Enk-OH	5.000	0.00	2
	Neuropeptide F	6.110	0.06	4
	desamino-nor-Y8Ra	7.270	0.10	3
30	(2DME) Y8Fa	9.200	0.01	3
	L-arginine	5.000	0.00	1
	D-arginine	5.000	0.00	1
	desipramine	5.000	0.00	1



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fenfluramine	5.000	0.00	1
harmine	5.000	0.00	1
levocabastine	5.000	0.00	1
ibogaine	5.000	0.00	1
ritanserine	5.000	0.00	1
a-MSH	5.000	0.00	1
Tyr-MIF-1	5.000	0.00	1
nociceptin	5.000	0.00	1
nocistatin	5.000	0.00	1
PMRFa	8.550	0.06	2
FTRF	7.870	0.10	2
FFRF	8.000	0.001	2

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Table 2

pKi for rat spinal cord membrane receptor binding

5	COMPOUND	MEAN	SEM	n
	NPFF (F-8-fa)	9.055	0.08	2
	(D-Tyr <sup>1</sup> - (NMe) Phe <sup>3</sup> ) NPFF	*9.724	0.25	4
	A18Fa	*9.000	0.21	2
	PQRFa	8.541	0.07	2
10	FMRFa	8.493	0.23	2
	Y8Fa	*9.189	0.06	2
	[Y <sup>9</sup> ] A18Fa	*8.502	0.01	2
	hPP	5.000	0.00	3
	fPP	*9.118	0.06	3
15	substance P	5.000	0.00	1
	Dynorphin A1-13	*5.700	0.50	2
	(3D) Y8Fa	9.123	0.12	4
	(2D) Y8Fa	*9.212	0.23	4
	CCK8	5.000	0.00	2
20	galanin	5.000	0.00	2
	dopamine	5.000	0.00	2
	naloxone	5.000	0.00	2
	CGRP	5.000	0.00	2
	AF-1	*7.563	0.47	2
25	AF-2	*7.965	0.24	2
	SchistFLRF	6.390	0.23	2
	Met-Enk-NH <sub>2</sub>	*8.400	0.08	4
	Met-Enk-OH	5.000	0.00	2
	Neuropeptide F	*8.100	0.10	3
30	desamino-nor-Y8Ra	7.51	0.07	3
	(2DME) Y8Fa	9.570	0.30	4
	L-arginine	5.000	0.00	1
	D-arginine	5.000	0.00	1

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	desipiramine	5.000	0.00	1
	fenfluramine	5.000	0.00	1
	harmine	5.000	0.00	1
	levocabastine	5.000	0.00	1
5	ibogaine	5.000	0.00	1
	ritanserine	5.000	0.00	1
	$\alpha$ -MSH	5.000	0.00	1
	Tyr-MIF-1	5.000	0.00	1
	nociceptin	5.000	0.00	1
10	nocistatin	5.000	0.00	1
	PMRFa	9.370	0.11	2
	FTRF	8.160	0.16	2
	FFRF	8.980	0.001	2

15

AF-1 = FMRF-like peptide

 $H_2N$ -Lys-Asn-Gln-Phe-Ile-Arg-Phe-NH<sub>2</sub>AF-2 H-Lys-His-Gln-Tyr-Leu-Arg-Phe-NH<sub>2</sub>Schisto(FLRFNH<sub>2</sub>) =

Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-amide

20

Met<sup>5</sup>, Arg<sup>6</sup>, Phe<sup>7</sup>- NH<sub>2</sub> = enkephalinamideMet<sup>5</sup>, Arg<sup>6</sup>, Phe<sup>7</sup>- OH = enkephalin

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Table 3

pKi of NPFF-related peptides at cloned human and rat NPFF receptors in 293 human embryonic kidney cells (HEK-293 cells)

	human		rat	
	NPFF1	NPFF2	NPFF1	NPFF2
	pKi $\pm$ SEM			
(D-Tyr <sup>1</sup> -(NMe)Phe <sup>5</sup> ) NPFF	8.1 $\pm 0.06$	8.5 $\pm 0.08$	8.8 $\pm 0.005$	8.7 $\pm 0.16$
fPP	5.9 $\pm 0.09$	7.4 $\pm 0.13$	5.4 $\pm 0.10$	7.3 $\pm 0.02$
FMRF amide	9.1 $\pm 0.19$	8.4 $\pm 0.02$	8.7 $\pm 0.01$	8.0 $\pm 0.02$
D-Met-FMRF amide	6.6 $\pm 0.24$	6.4 $\pm 0.03$	6.2 $\pm 0.09$	6.2 $\pm 0.03$
A18Fa	7.2 $\pm 0.11$	8.9 $\pm 0.13$	7.5 $\pm 0.14$	8.2 $\pm 0.007$
PQRFa	7.4 $\pm 0.45$	7.6 $\pm 0.05$	7.6 $\pm 0.05$	7.6 $\pm 0.004$
BIBP 3226	6.9 $\pm 0.04$	5.9 $\pm 0.04$	7.6 $\pm 0.04$	5.8 $\pm 0.02$

Table 4

Activation of intracellular calcium mobilization by COS-7 cells expressing human NPFF receptors and Gq/Gz chimera.

5	Compound	NPFF1		NPFF2	
		pEC50	% of NPPF Response	pEC50	% of NPPF Response
10	NPFF	7.8 ± 0.10	100	8.7 ± 0.02	100
	fPP	< 5.0	0	6.7 ± 0.07	78
	PQRF	6.7 ± 0.02	93	7.0 ± 0.04	94
15	amide				

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Localization

Detection of mRNA coding for rat NPFF1 receptors: mRNA was isolated from multiple tissues (Table 3) and assayed as described. The distribution of mRNA encoding rat NPFF1 receptors is widespread throughout the central nervous system, and structures associated with the nervous system (Table 3, Figures 19, 20). The highest levels of rNPFF1 mRNA are found in the hypothalamus and the pituitary gland. The protected segment seen with mRNA isolated from the pituitary, adrenal gland and ovary is considerably shorter than that seen in other tissue (Figure 20) and indicates the possibility of splice variants of this receptor. Peripheral organs contain little or no mRNA encoding rNPFF1 with the exception of the testes, ovary, the adrenal medulla and the adrenal cortex. There is good correlation between the distribution determined by RT-PCR and RPA (Table 3, Figures 19, 20). RT-PCR detected rat NPFF1 in more areas than RPA as it is a more sensitive technique.

High levels of mRNA encoding NPFF receptors in the hypothalamus and pituitary, with relatively low expression in most of the other regions assayed implicates this receptor in neuroendocrine control, as well as the control of feeding and metabolic regulation. Its presence in other areas, including the spinal cord, medulla and dorsal root ganglia implicate NPFF receptors as a potential modulator of pain and/or sensory transmission. Low levels in the hippocampal formation indicate a possible role in learning and memory.

Table 5

Summary of distribution of mRNA coding for rat NPFF1  
receptors

Tissue	RT-PCR	Ribonuclease protection assay (RPA)	Potential applications
adrenal cortex	+	+	regulation of steroid hormones
adrenal medulla	+	++	regulation of epinephrine release
urinary bladder	-	-	urinary incontinence
duodenum	+/-	-	gastrointestinal disorders
heart	+/-	-	cardiovascular indications
kidney	+	-	electrolyte balance, hypertension
liver	+/-	-	diabetes
lung	+/-	-	respiratory disorders, asthma
ovary	+	+	reproductive function
pancreas	+/-	NA	diabetes, endocrine disorders
spleen	+/-	-	immune disorders
stomach	+/-	-	gastrointestinal disorders
striated muscle	+/-	-	musculoskeletal disorders

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testicle	+/-	+	reproductive function
uterus	+/-	-	reproductive function
vas deferens	-	-	reproductive function
whole brain	+++		
spinal cord	++	++	analgesia, sensory modulation and transmission
amygdala	+++	+/-	
caudate/ putamen	++	-	modulation of dopaminergic function
cerebellum	+++	+	motor coordination
cerebral cortex	++	+	Sensory and motor integration, cognition
DRG	+	+	sensory transmission
hippocampus	+++	+	cognition/memory
hypothalamus	+++	+++	appetite/obesity, neuroendocrine regulation
medulla	++	++	analgesia, motor coordination
olfactory bulb	++	NA	olfaction
pituitary	+++	+++	Endocrine/neuro- endocrine regulation
substantia nigra	+++	++	Modulation of dopaminergic function



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superior cervical ganglion	+	-	modulation of sympathetic innervation
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NA = not assayed

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Localization of mRNA coding for hNPFF2 receptors using RT-PCR

**Detection of mRNA coding for hNPFF2 receptors**

5 mRNA was isolated from multiple tissues (Table 4) and assayed as described. The distribution of mRNA encoding hNPFF2 receptors is widespread throughout all regions assayed. (Table 4, Figure 21).

Table 6

Distribution of mRNA coding for hNPFF2 receptors

5	Region	hNPFF2	Potential Implications
	liver	++	Diabetes
	kidney	++	Hypertension, electrolyte balance
	Lung	++	Respiratory disorders, asthma
	heart	++	Cardiovascular indications
10	stomach	++	Gastrointestinal disorders
	small intestine	++	Gastrointestinal disorders
	spleen	++	Immune function
	pancreas	++	Diabetes, endocrine disorders
	striated muscle	++	Musculoskeletal disorders
15	pituitary	++	Endocrine/neuroendocrine regulation
	whole brain	++	
	amygdala	++	Depression, anxiety, mood disorders
	hippocampus	++	Cognition/memory
	spinal cord	++	Analgesia, sensory modulation and transmission
20	cerebellum	++	Motor coordination
	thalamus	++	sensory integration
	substantia nigra	++	Modulation of dopaminergic function and motor coordination
	caudate	++	Modulation of dopaminergic function
	fetal brain	++	Developmental disorders

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fetal lung	++	Developmental disorders
fetal kidney	++	Developmental disorders
fetal liver	++	Developmental disorders
HEK-293 cells	+	
HeLa cells	-	
Jurkat cells	-	

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Localization of mRNA coding for human and rat NPFF.Results

mRNA was isolated from multiple tissues (listed in Table 7) and assayed as described.

Human NPFF1

Quantitative RT-PCR using a fluorigenic probe demonstrated mRNA encoding human NPFF1 RNA to be localized in highest abundance in CNS tissue. All CNS tissues assayed demonstrate moderate levels of NPFF1 RNA. The broad distribution of NPFF1 mRNA implies a modulatory role in multiple systems within the CNS. Highest levels are found in the spinal cord, hippocampus, amygdala, thalamus and hypothalamus. High levels in the spinal cord and thalamus imply an important role in sensory transmission or modulation (including nociception). The hippocampal formation and amygdala also contain high levels of NPFF1 mRNA. Localization to these structures support the hypothesis that NPFF is involved in the modulation of learning and memory as well as having a role in the regulation of fear, mood, and may provide a target for the treatment of depression, anxiety, phobias and mood disorders.

NPFF1 mRNA is also expressed in the hypothalamus in moderate amounts. This suggests a role in neuroendocrine regulation, regulation of circadian rhythms, regulation of appetite/feeding behavior and other functions that are modulated by the hypothalamus. NPFF1 mRNA is also expressed, although at somewhat lower levels, in the basal ganglia. The caudate-putamen, and substantia nigra both express moderate levels of NPFF1 mRNA. Localization to these regions implies a role in regulation of dopaminergic systems, and may provide a therapeutic target for treatment of movement disorders such as Parkinsons disease or tardive dyskinesia. The cerebellum also contains substantial

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amounts of NPFF1 mRNA indicating a role in the control of movement.

5 Fetal brain, although expressing NPFF1 mRNA, does so in much lower levels than that found in the adult. There is a five-fold difference in mRNA levels between fetal and adult brain. It is not known at this time if the developmental regulation is global within the CNS or restricted to selected regions. The time course of this increase has not  
10 been examined and would be important in understanding the function of this receptor.

In peripheral tissue, all tissues assayed expressed measurable NPFF1 mRNA levels. However, levels in peripheral  
15 tissue are much lower than those found in the CNS. The peripheral tissues expressing the highest levels of NPFF1 mRNA are spleen, lung and fetal lung. Levels in these tissues are more than 10 fold lower than that detected in the highest CNS regions. Others tissues assayed contain low  
20 levels of NPFF1 mRNA as indicated in Table 7.

In summary, the distribution of human NPFF1 mRNA implies broad regulatory function in the CNS, most notably in sensory transmission, modulation of the limbic system,  
25 modulation of feeding/circadian rhythms, and modulation of extrapyramidal motor systems. Its presence, albeit at low levels in peripheral tissues implies of broad regulatory role in multiple organ systems.

30

#### Human NPFF2

35 Unlike the distribution of human NPFF1 mRNA, which is expressed primarily in the CNS, the highest levels of NPFF2 RNA are found in the placenta. Expression in the placenta is four-fold higher than any other tissue assayed (Table 7).

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Presence of high levels NPFF2 receptor mRNA in the placenta indicates a role in gestational regulation and possible gestational abnormalities. It is not known at this time, whether NPFF2 mRNA is present at all stages of development, or which cells in the placenta express these receptors. Other tissues expressing NPFF2 mRNA include the small intestine, pituitary and spleen. RNA levels in the placenta are 20 fold higher than in these organs.

Within the CNS, highest levels of NPFF2 mRNA expression are found in the amygdala, caudate-putamen, and the hippocampal formation. These regions also express high levels of NPFF1 mRNA. As with NPFF1, localization to limbic structures supports the hypothesis that NPFF is involved in the modulation of learning and memory as well as having a role in the regulation of fear, mood, and may provide a target for the treatment of depression, anxiety, phobias and mood disorders. Localization to the caudate/putamen implies regulation of dopaminergic systems and a role in the regulation of extrapyramidal motor systems. Other areas assayed are listed in Table 7.

In summary, human NPFF2 mRNA is broadly distributed in both CNS and peripheral tissue. This implies broad regulatory functions in multiple organ systems. High levels in the placenta indicate a significant role in gestational physiology. Within the CNS, its implied function is modulation of the limbic system and extrapyramidal motor systems. Its presence, albeit at low levels in multiple tissues implies a broad modulatory role involving multiple physiological modalities.

#### Rat NPFF1

As with the human NPFF1 receptor mRNA, highest levels of rat NPFF1 RNA are found in central nervous system structures. Highest levels are found in the hypothalamus, amygdala, and the substantia nigra. All CNS structures assayed express

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rNPFF1 RNA (Table 8).

5 The high levels of NPFF1 mRNA expressed in the hypothalamus indicate a role in neuroendocrine regulation, regulation of circadian rhythms, regulation of appetite and other functions that are modulated by the hypothalamus. High levels in the amygdala and other limbic (or limbic related) structures suggest a role in modulation of mood, fear, phobia, anxiety and may provide a therapeutic target for the treatment of depression and other neuropsychiatric disorders.

15 The presence of lower levels of NPFF1 RNA in other areas such as the hippocampal formation, spinal cord, medulla, caudate-putamen, cerebral cortex, cerebellum and other areas suggests diverse functions as suggested in Table 8.

20 The tissue showing the highest levels of NPFF1 mRNA outside the CNS is the testes. Levels in the testes are more than approximately half of the levels found in the hypothalamus, and containing approximately the same levels as those found in the amygdala, substantia nigra, and olfactory bulb (see Table 8). This strongly suggests a role in endocrine regulation or reproductive function. Other peripheral tissues showing moderate amounts of NPFF1 mRNA are listed in Table 8.

#### Rat NPFF2

30 As with rat NPFF1, high levels of rat NPFF2 mRNA are found in CNS structures. Highest levels are found in the spinal cord and medulla. Localization to these structures as well as moderate levels in the dorsal root and trigeminal ganglia, strongly suggest a role in sensory transmission (or modulation) including nociceptive stimuli. In addition to the above, there are also moderate levels of NPFF2 RNA localized to the retina. This suggests a role in modulation of visual stimuli or circadian rhythms.



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Other CNS regions expressing high levels of NPFF2 RNA include the hypothalamus, substantia nigra and amygdala. The high levels of NPFF2 mRNA expressed in the hypothalamus indicate a role in neuroendocrine regulation, regulation of circadian rhythms, regulation of appetite and other functions that are modulated by the hypothalamus. High levels in the amygdala suggests a role in modulation of mood, fear, phobia, anxiety and may provide a therapeutic target for the treatment of depression and other neuropsychiatric disorders.

The tissue expressing the highest levels of NPFF2 mRNA outside the CNS is the heart. NPFF2 RNA levels in the heart are comparable to those found in many CNS structures. The heart expresses similar levels of NPFF2 RNA as the spinal cord, medulla, hypothalamus, or substantia nigra. Another tissue expressing moderate levels of NPFF2 mRNA is the aorta. This distribution strongly implies regulation of cardiovascular function, perhaps by innervation from brain stem autonomic centers. It is not known if the NPFF2 mRNA is localized to myocytes within the heart or if they are localized on the conductance fibers, smooth muscle, or endothelial cells.

Lower levels of NPFF2 mRNA are localized in multiple tissues though the body as listed in Table 8. The localization of NPFF2 mRNA to every tissue assayed indicated that this receptor may have broad regulatory roles in multiple systems.

In summary: The distribution of rat NPFF1 implies a role in limbic function as described, and the distribution of NPFF2 implies a role in sensory transmission or modulation. The broad distribution of both of these receptors in the central nervous system as well as in peripheral organs, implies a broad regulatory role in multiple physiological systems.

Table 7

Distribution of mRNA coding for human NPFF receptors using qRT-PCR

(mRNA encoding NPFF is expressed as % of highest

5 expressing tissue: spinal cord for NPFF 1 and placenta for NPFF2 + SEM)

	Region	h-NPFF1	h-NPFF2	Potential applications
	heart	0.21 +0.03	0.39 +0.21	Cardiovascular indications
10	kidney	0.67 +0.11	0.83 +0.13	Hypertension, electrolyte balance
	liver	0.35 +0.07	0.21 +0.04	Diabetes
	lung	6.96 +0.56	0.71 +0.09	Respiratory disorders, asthma
	pancreas	0.23 +0.06	0.53 +0.09	Diabetes, endocrine disorders
	pituitary	2.46 +0.32	4.65 +0.47	Endocrine/ neuroendocrine regulation
15	placenta	0.23 +0.03	100 +13.20	Gestational abnormalities
	small intestine	2.74 +0.10	4.39 +0.17	Gastrointestinal disorders
	spleen	8.08 +0.55	3.81 +0.28	Immune disorders
	stomach	0.55 +0.06	0.095 +0.14	Gastrointestinal disorders
	striated muscle	1.22 +0.16	0.78 +0.12	Musculoskeletal disorders
20	amygdala	43.52 +4.35	27.24 +1.78	Depression, phobias, anxiety, mood disorders
	caudate-putamen	19.04 +0.75	9.30 +1.12	Modulation of dopaminergic function
	cerebellum	20.48 +2.14	trace	Motor coordination
	hippocampus	44.56 +5.55	7.39 +0.75	Cognition/memory

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5	hypothalamus	20.65 +0.97	1.58 +0.02	appetite/obesity, neuroendocrine regulation
	spinal cord	100 +4.97	1.32 +0.07	Analgesia, sensory modulation and transmission
	substantia nigra	13.36 +0.81	0.57 +0.06	Modulation of dopaminergic function. Modulation of motor coordination.
	thalamus	29.84 +3.75	2.24 +0.27	Sensory integration disorders
	whole brain	21.28 +1.00	7.89 +1.12	
10	fetal brain	4.24 +0.33	0.69 +0.08	Developmental disorders
	fetal lung	6.01 +0.89	0.37 +0.08	Developmental disorders
	fetal kidney	1.89 0.23	2.86 +0.31	Developmental disorders
	fetal liver	trace	0.54 +0.06	Developmental disorders

Table 8

## Summary of distribution of mRNA coding for rat NPFF receptors

(mRNA encoding NPFF is expressed as % of highest expressing tissue: hypothalamus for NPFF 1 and placenta for NPFF2 + SEM)

Tissue	rNPFF1	rNPFF2	Potential applications
adipose	2.56 +0.24	11.72 +3.17	metabolic disorders
adrenal cortex	2.98 +0.35	4.70 +0.44	regulation of steroid hormones
adrenal medulla	16.84 +1.23	trace	regulation of epinephrine release
amygdala	57.09 +10.25	41.65 +5.31	depression, phobias, anxiety, mood disorders
aorta	1.23 +0.24	23.83 +3.70	cardiovascular indications
celiac plexus	3.60 +0.14	12.15 +1.25	modulation of autonomic function
cerebellum	17.33 +1.69	10.41 +1.51	motor coordination
cerebral cortex	21.72 +0.78	10.99 +2.29	Sensory and motor integration, cognition
choroid plexus	24.82 +1.10	29.54 +6.92	regulation of cerebrospinal fluid
colon	trace	8.38 +2.72	gastrointestinal disorders
dorsal root ganglia	2.77 +0.46	38.26 +3.47	sensory transmission
duodenum	trace	5.28 +0.37	gastrointestinal disorders
heart	3.19 +0.30	82.32 +7.97	cardiovascular indications
hippocampus	20.27 +1.63	8.28 +2.41	cognition/memory
hypothalamus	100 +6.15	84.26 +11.01	appetite/obesity, neuroendocrine regulation

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kidney	1.03 +0.23	20.44 +1.36	electrolyte balance, hypertension
liver	1.82 +0.31	3.20 +0.42	diabetes
lung	3.72 +0.29	15.88 +4.35	respiratory disorders, asthma
medulla	22.44 +2.21	92.01 +6.49	analgesia, motor coordination
nucleus accumbens	34.75 +0.78	10.85 +1.60	regulation of dopaminergic function, drug addiction, neuropsychiatric disorders
olfactory bulb	40.96 +4.01	9.83 +4.53	olfaction
ovary	13.74 +1.85	12.35 +2.59	reproductive function
pancreas	trace	trace	diabetes, endocrine disorders
pineal	trace	4.12 +0.95	regulation of melatonin release
pituitary	23.58 +1.81	33.90 +1.94	endocrine/neuroe ndocrine regulation
retina	14.15 +0.97	40.19 +2.48	visual disorders
salivary gland	trace	32.93 +7.48	
spinal cord	24.00 +1.41	100 +5.91	analgesia, sensory modulation and transmission
spleen	trace	trace	immune disorders
stomach	trace	13.90 +0.69	gastrointestinal disorders
striated muscle	trace	trace	musculoskeletal disorders
striatum	17.33 +1.69	16.37 +4.59	modulation of dopaminergic function, motor disorders

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	substantia nigra	48.82 +5.54	66.83 +8.45	modulation of dopaminergic function, modulation of motor coordination
	testes	42.61 +4.71	4.31 +0.68	reproductive function
5	thalamus	3.14 +0.25	14.92 +1.92	sensory integration disorders
	thymus	trace	11.53 +2.92	immune disorders
10	trigeminal ganglia	16.09 +0.14	56.82 +2.33	sensory transmission
	urinary bladder	trace	15.79 +1.39	urinary incontinence
	uterus	trace	trace	reproductive disorders
	vas deferens	trace	trace	reproductive function
	whole brain	21.49 +1.88	23.83 +2.97	.

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Localization of NPFF Receptor Subtypes in the rat CNSTelencephalon

5 The cerebral cortex and the amygdala displayed  
[<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe) Phe<sup>3</sup>] NPFF binding just above background for  
both the NPFF1 and NPFF2 receptors.

10 In the basal ganglia the globus pallidus was devoid of any  
specific binding. [<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe) Phe<sup>3</sup>] NPFF binding  
related to the NPFF2 receptor was discretely located in the  
dorsolateral caudate-putamen and was completely displaced by  
frog PP. NPFF1 binding sites were evident above background  
15 in the accumbens nucleus. Within the septum there was a  
rostrocaudal gradient in binding sites related to NPFF1.  
The greatest density of binding was observed in the more  
caudal laterodorsal and the intermediate lateral septal  
nuclei, while rostrally a moderate density was observed.  
Additionally, moderate NPFF1 binding was detected in the  
medial septum. See Table 9.

20

Diencephalon

In the thalamus the majority of [<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe) Phe<sup>3</sup>] NPFF  
binding was related to the NPFF2 receptor subtype. NPFF2  
receptors were detected in the paraventricular and  
25 paratenial nuclei, as well as in the reticular,  
laterodorsal, anterior pretectal, and parafascicular  
thalamic nuclei. A significant density of NPFF1 binding  
sites were detected in the anterodorsal thalamic nucleus  
with lower expression in the paraventricular, central medial  
30 and ventral nuclei. In the epithalamus, NPFF2 receptors  
were present in the lateral habenula. See Table 9.

35 In the hypothalamus, a rostrocaudal gradient of  
[<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe) Phe<sup>3</sup>] NPFF binding to the NPFF2 receptor was  
observed in the lateral hypothalamus with the highest  
density of binding rostrally. The medial mammillary nucleus  
also contained considerable NPFF2 receptor binding while  
moderate binding was seen in the lateral anterior

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hypothalamus. A lower expression of NPFF2 binding sites was observed in the lateroanterior hypothalamus. NPFF1 binding sites were difficult to determine in the hypothalamus due to high background levels and the possible underestimation of NPFF1 binding densities (see Discussion), however, NPFF1 receptor binding sites were detectable in the tuber cinereum. See Table 9.

The hippocampal formation did not exhibit any specific [<sup>125</sup>I] [D-Tyr<sup>1</sup>-(NMe)Phe<sup>3</sup>]NPFF binding in Ammon's horn, although, a moderate number of NPFF1 binding sites were observed in the ventral dentate gyrus. In other related limbic structures, NPFF1 receptor binding sites were detected in the bed nucleus of the stria terminalis and the pre/parasubiculum appeared to contain both NPFF1 and NPFF2 receptors. See Table 9.

#### Mesencephalon

[<sup>125</sup>I] [D-Tyr<sup>1</sup>-(NMe)Phe<sup>3</sup>]NPFF binding to NPFF2 receptors was identified in the anterior pretectal nucleus and displayed a dorsal to ventral gradient with the highest density dorsally. NPFF2-receptor binding was also observed in the medial pretectal nucleus, posterior intralaminar thalamic nucleus, interstitial nucleus of mlf, substantia nigra, compact part, interpeduncular nucleus, rostral and caudal linear nuclei of raphe, dorsal and median raphe nuclei, retrorubral field, B9 5-hydroxytryptamine cells, medial and lateral parabrachial nuclei, and the microcellular tegmental nucleus. Moderate [<sup>125</sup>I] [D-Tyr<sup>1</sup>-(NMe)Phe<sup>3</sup>]NPFF binding to the NPFF2 receptor was visible in the dorsal and ventral periaqueductal gray and there was a very weak signal in the ventral periaqueductal gray related to the NPFF1 receptor. The superior colliculus, pontine nuclei, and the caudal linear raphe nucleus contained NPFF1 receptor binding sites, while the parabrachial nucleus exhibited NPFF1 binding sites just above background. See Table 9.

#### Rhombencephalon (Pons/Medulla)



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NPFF2 receptor binding sites were evident in the medial vestibular, spinal trigeminal, gigantocellular reticular, Barrington's and ventral cochlear nuclei, in addition to the nucleus of the solitary tract. The highest density of NPFF2 binding sites in the rhombencephalon was seen in the region of the facial nerve in the vicinity of the A5 noradrenaline cells. Throughout the pons and medulla there was a low homogeneous ligand binding just above background which appeared to be related to the NPFF2 receptor. NPFF1 binding sites were detectable in Barrington's nucleus, the nucleus of the solitary tract, principal trigeminal nucleus and throughout the reticular formation. See Table 9.

#### Spinal cord

The dorsal horn displayed the greatest number of [<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe) Phe<sup>3</sup>]NPFF binding sites in the spinal cord. Ligand binding in the substantia gelatinosa and lamina X was attributed to the NPFF2 receptor. NPFF1 binding sites were evident in the spinal cord ventral horn. See Table 9.

Table 9

Distribution of NPFF1 and NPFF2 receptors in the rat CNS

Region	rNPFF1	rNPFF2	Potential Application
<b>Telencephalon</b>			
5 cerebral cortex	+	+	Cognition, sensory and motor integration
amygdala	+	+	Emotion and memory, social behaviors, modulation of autonomic and neuroendocrine systems
10 vertical diagonal band	+	-	Memory, modulation of cholinergic transmission
horizontal diagonal band	+	-	Memory, modulation of cholinergic transmission
globus pallidus	-	-	
15 caudate-putamen	-	+	Sensory/motor integration
accumbens n.	+	-	Modulation of dopaminergic function
20 lateral septal n., dorsal	+	+	Modulation of higher cognitive functions, emotions, and autonomic regulation
medial septal n.	+	-	Cognitive enhancement via cholinergic system

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	Diencephalon			
	para-ventricular thal. n.	+	+	
5	central medial thalamic n.	+	+	
	paratenial thalamic n.	-	+	Modulation of information to the medial prefrontal cortex
10	anterodorsal thalamic n.	+	+	Modulation of motor information to the cerebral cortex/Eye movement
	reticular thalamic n.	-	+	Alertness/sedation
15	laterodorsal thalamic n.	-	+	Emotional expression
	para-fascicular thal. n.	-	+	Motor and behavioral responses to pain
20	latero-anterior hypothal.	-	+	
	lateral hypothalamus	-	+	Ingestive behavior, modulation of pain
25	tuber cinereum	+	+	
	supra-chiasmatic n.	-	+	Circadian rhythm

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	medial mammillary n.	-	+	Integration of autonomic or limbic-related functions with movement
	lateral habenular n.	-	+	
5	<b>Hippocampal formation</b>			
	Ammon's horn	-	-	
	ventral dentate gyrus	+	-	Cognition/Memory
10	bed n. stria terminalis	+	+	<b>Central autonomic system</b>
	pre/para- subiculum	+	+	Modulation of memory aquisition
	<b>Mesencephalon</b>			
15	anterior pretectal n.	-	+	Mediate visual reflexes/nociception
	medial pretectal n.	-	+	
	post. intra- laminar n.	-	+	
20	interstitial n. of mlf	-	+	
	superior colliculus	+	-	Modulation of visual information/spatial localization
25	peri- aqueductal gray	+	+	Analgesia

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	substantia nigra, compact part	-	+	Modulation of DA function/Motor coordination
5	substantia nigra, reticular part	-	-	
	inter-peduncular n.	+	+	Analgesia
10	rostral linear n. raphe	-	+	
	caudal linear n. raphe	+	+	
15	red n.	-	-	
	microcellular tegmental n.	-	+	
	dorsal raphe n.	-	+	Analgesia
20	median raphe n.	-	+	
	locus coeruleus	-	-	
25	Barrington's n.	+	+	Pontine micturition center-urinary bladder function
	A5 noradrenergic cell group	-	+	Control of autonomic functions, modulating the perception of pain; blood pressure

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	Rhomb- encephalon (Pons/ Medulla)			
5	medial vestibular n.	-	+	Maintenance of balance and equilibrium, Modulating auditory information
	n. of solitary tract	+	+	Modulation of gustatory and somatosensory information
10	parabrachial n.	+	+	Modulation of visceral sensory information
	spinal trigeminal n.	-	+	Nociception
	cerebellum	-	-	
15	giganto- cellular reticular n.	+	+	Nociception/Analgesia
	ventral cochlear n.	-	+	Modulation of auditory information
20	<b>Spinal cord</b>			
	dorsal horn	-	+	Nociception/Analgesia
	ventral horn	+	-	
	lamina X	-	+	Nociception, sensory-visceral reflex arc

25

### Discussion

The anatomical distribution of the NPFF1 and NPFF2 receptors in the rat CNS was determined by receptor autoradiography using [<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe)Phe<sup>3</sup>]NPFF at 0.05 nM and making use of subtype selective displacers to directly visualize the individual receptors, NPFF1 and NPFF2. The radioligand exhibits a somewhat higher affinity for the rat NPFF2 subtype ( $K_d = 0.22$  nM) relative to the rat NPFF1 subtype ( $K_d = 0.65$  nM). Thus the data may reflect an approximately threefold underestimate of the NPFF1 receptor density relative to that for the NPFF2 subtype. [<sup>125</sup>I] [D-Tyr<sup>1</sup>- (NMe)Phe<sup>3</sup>]NPFF binding to the NPFF1 receptor was defined as the frog PP-insensitive binding, as this compound is highly selective for NPFF2 [ $pK_i = 7.3 \pm 0.02$  at rat NPFF2 and  $5.4 \pm 0.010$  at rat NPFF1 (Table 3)]. Binding to the NPFF2 receptor was defined as the BIBP 3226-insensitive binding, as BIBP 3226 is highly selective for the NPFF1 receptor [ $pK_i = 7.6 \pm 0.04$  at rat NPFF1 and  $5.8 \pm 0.02$  at rat NPFF2] (Table 3). The results suggest that while both NPFF1 and NPFF2 receptors are present in the rat CNS, the NPFF2 receptor appears to be the predominantly expressed receptor. NPFF1 and NPFF2 receptors are discretely localized in a number of brain nuclei.

NPFF1 receptors were observed to be in cholinergic forebrain regions, namely the nucleus of the diagonal band, the medial and lateral septal nuclei, and the ventral dentate gyrus. NPFF1 binding sites were also detected in the superior colliculus and the spinal cord ventral horn. NPFF2 receptors were found to be present in numerous nuclei in the diencephalon, namely the reticular and laterodorsal thalamic nuclei, the suprachiasmatic, lateroanterior, lateral, and medial mammillary hypothalamic nuclei. Caudally, NPFF2 receptors were found in the compact part of the substantia nigra, periaqueductal gray and various raphe nuclei. In all levels of the spinal cord, the dorsal horn and lamina X contained NPFF2 receptor binding sites.

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NPFF-like immunoreactivity (NPFF-LI) has been described in the rat brain (74, 21). The distribution of NPFF-LI in the rat CNS is very limited, the highest levels of immunoreactivity were observed in the hypothalamus and the spinal cord. NPFF-LI neurons were identified in the medial hypothalamus and nucleus of the solitary tract, while immunoreactive fibers were evident in the lateral septal nucleus, amygdala, the lateral hypothalamus, median eminence, bed nucleus of the stria terminalis, nucleus of the diagonal band, nucleus of the solitary tract, the ventral medulla and the trigeminal complex. NPFF-LI cells and terminals, as well as the mRNA for both NPFF1 and NPFF2 (Table 8), have been reported to be present in the substantia gelatinosa and lamina X at all levels of the spinal cord of rats (75, 21). The distribution of the NPFF1 and NPFF2 receptor binding sites correlates well with the distribution of the NPFF-LI neurons and terminals. Additionally, the distribution of the NPFF1 and NPFF2 receptors is concordant with previous reports of the anatomical distribution of NPFF binding sites obtained using [<sup>125</sup>I] [D-Tyr<sup>1</sup>-(NMe)Phe<sup>3</sup>]NPFF (35) and [<sup>125</sup>I]Y8Fa (76).

#### Potential Application

NPFF-like peptides have been associated with pain mechanisms, opioid tolerance, autonomic functions, memory and neuroendocrine regulation.

The anatomical distribution of NPFF-LI, NPFF2 mRNA and NPFF2 receptor binding sites supports the idea of a role for the NPFF2 receptor in the regulation of pain and analgesia, perhaps by modulating the effects of the endogenous opioid peptides. NPFF has been shown to attenuate the analgesic effects of morphine after intrathecal and intraventricular injection (77) and the localization suggests that this effect be mediated by the NPFF2 receptor. NPFF-LI in the spinal cord is thought to be mostly of intrinsic origin and NPFF-LI cells in rostral regions of the brain do not send descending fibers to the spinal cord (75). Additionally, no



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NPFF-LI is found in the dorsal root ganglia, and dorsal rhizotomy does not affect NPFF-LI in the dorsal spinal cord (27, 21). NPFF2 mRNA has been identified in DRGs, and this localization might imply that NPFF2 receptors are located on the primary afferent terminals, possibly mediating neurotransmitter release. NPFF-LI is concentrated in lamina I/II, the projection site for primary afferent terminals, a region that contains the highest density of NPFF2 binding sites. In the substantia gelatinosa primary afferents also make contact with large NPFF-LI nociceptive neurons which in turn project rostrally to the mesencephalon and thalamus, possibly playing a role in the autonomic and affective responses to pain. NPFF2 mRNA has been identified in the spinal cord supporting a role for this receptor in the ascending pain pathway. In lamina X there are NPFF-LI fibers possibly related to descending projections from cells originating around the central canal. Thus, the NPFF2 receptor may also be involved in sensory-visceral reflex arcs.

To further strengthen the concept that the NPFF2 receptor may be involved in nociceptive processing, NPFF2 binding sites were localized in a variety of brain regions known to be involved in nociception and pain modulation, namely the spinal trigeminal nucleus, parabrachial nucleus, gigantocellular reticular nucleus, A5 noradrenergic cell group, dorsal raphe nucleus, periaqueductal gray, lateral hypothalamus, and the parafascicular thalamic nucleus. Injection of an anterograde trace (PHA-L) into the intermediomedial hypothalamus, a site of NPFF-ir cell bodies, supports the concordance between the NPFF2 binding site distribution and NPFF-ir terminals in many of these regions (80). Furthermore, NPFF2 mRNA has been identified in the hypothalamus and medulla. NPFF2 mRNA was also detected in the trigeminal ganglion which is most likely one of the sources of NPFF2 receptors found in the spinal trigeminal nucleus (Table 8).

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There is some discordance between the localization of NPFF1 mRNA and NPFF1 binding sites in the spinal cord. While there is no autoradiographic evidence for NPFF1 binding sites in the dorsal horn, NPFF1 mRNA has been identified in the spinal cord and DRG (Table 8). This discrepancy might be explained by the expression of the NPFF1 receptor on the peripheral terminal projections of the DRG cells or on the projections of spinal cord neurons to the ventral horn or more rostrally in the brainstem and thalamus. The localization of NPFF1 mRNA in both spinal cord and DRG and with NPFF1 receptor binding sites in the ventral horn is consistent with a potential role for the NPFF1 receptor in the processing of nociceptive information.

NPFF-LI fibers are present in several limbic system-related structures, namely the hippocampus, lateral septal nucleus, accumbens nucleus, nucleus of the diagonal band, and bed nucleus of the stria terminalis. The NPFF1 receptor is expressed in these regions. Furthermore, NPFF1 mRNA has been detected in the accumbens nucleus, amygdala, and hippocampal formation. On the basis of this localization, a role for the NPFF1 receptor may be to regulate learning and memory and the emotional states of fear and anxiety (78). Kavaliers and Colwell (79) have shown that mice receiving icv injections of IgG from NPFF antiserum acquire spatial tasks more slowly and perform more poorly, while icv NPFF resulted in better acquisition of memory. The effect may be associated with the hypothalamo-limbic connections containing NPFF (80).

A role for NPFF receptors in regulating sensory information might be indicated by their presence in the relay nuclei of several sensory pathways. It appears that both of the receptors may participate in the modulation of the visual system. NPFF1 receptor binding sites were observed in the superior colliculus while NPFF2 receptor binding sites were detected in the suprachiasmatic nucleus. Both of these regions receive afferents from the retina that contains mRNA

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for NPFF1 and NPFF2. The possibility that the NPFF2 receptor might play a modulatory role in circadian rhythms is supported by the localization of NPFF2 binding sites in the suprachiasmatic nucleus. The suprachiasmatic nucleus receives direct input from the retina and is thought to be responsible for the maintenance of circadian rhythms. In the auditory system the NPFF2 receptor appears to be a possible modulator. The NPFF2 receptor is present in the cochlear and medial vestibular nuclei.

The identification of NPFF receptor binding sites and mRNA for NPFF1 and NPFF2 in various components of the basal ganglia, namely, the accumbens nucleus, the substantia nigra, compact part, and the caudate-putamen, suggests that NPFF receptors may be involved in regulation of the dopaminergic system, although they are not found on dopaminergic neurons (81). Ibotenic acid lesion studies have shown that NPFF receptors in the substantia nigra, compact part are on afferent fibers, and thus may indirectly influence the mesocorticolimbic system. NPFF2 receptors were identified in the dorsolateral caudate-putamen, an region which represents the target area for the somatosensory cortex and may be involved in sensorimotor integration.

Some of the highest NPFF-LI in the brain was observed in the hypothalamus, one of the main loci for NPFF-LI cell bodies (74). The lateral hypothalamus is involved in catecholaminergic and serotonergic feeding systems and plays a role in circadian feeding and spontaneous activity. The localization of NPFF2 receptor binding sites and mRNA in this region suggests that the NPFF2 receptor may be involved in the regulation of ingestive behavior. Some of the NPFF2 receptor binding sites in the hypothalamus may be located presynaptically on projections from the amygdala since NPFF2 mRNA has been detected in the amygdala (Table 8). In addition, NPFF1 mRNA was detected in the amygdala and hypothalamus (Table 8), suggesting that NPFF1 receptors may

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also be involved in the regulation of ingestive behaviors. While NPFF1 binding sites were not evident in the hypothalamus, there was a low density of NPFF1 binding sites seen in the amygdala. The lateral parabrachial and nucleus  
5 of the solitary tract are two other brain regions involved in the regulation of feeding that contain NPFF1 and NPFF2 receptor binding sites. The origin of dense immunoreactive terminals in these regions is thought to be from the hypothalamus where NPFF1 and NPFF2 mRNA have been found,  
10 further supporting a potential role for both receptors in ingestive behaviors.

The cloning of the gene encoding NPFF receptors has provided the means to explore their physiological roles by  
15 pharmacological characterization, and by Northern and in situ mapping of its mRNA distribution. Further, the availability of the DNA encoding the NPFF receptors will facilitate the development of antibodies and antisense technologies useful in defining the functions of the gene  
20 products in vivo. Antisense oligonucleotides which target mRNA molecules to selectively block translation of the gene products in vivo have been used successfully to relate the expression of a single gene with its functional sequelae. Thus, the cloning of these receptor genes provides the means  
25 to explore their physiological roles in the nervous system and elsewhere, and may thereby help to elucidate structure/function relationships within the GPCR superfamily.

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What is claimed is:

1. An isolated nucleic acid encoding a mammalian NPFF receptor.
- 5 2. The nucleic acid of claim 1, wherein the nucleic acid is DNA.
3. The DNA of claim 2, wherein the DNA is cDNA.
- 10 4. The DNA of claim 2, wherein the DNA is genomic DNA.
5. The nucleic acid of claim 1, wherein the nucleic acid is RNA.
- 15 6. The nucleic acid of claim 1, wherein the mammalian NPFF receptor is a NPFF1 receptor.
7. The nucleic acid of claim 6, wherein the mammalian NPFF1 receptor is a rat NPFF1 receptor.
- 20 8. The nucleic acid of claim 6, wherein the mammalian NPFF1 receptor is a human NPFF1 receptor.
- 25 9. The nucleic acid of claim 1, wherein the mammalian NPFF receptor is a NPFF2 receptor.
10. The nucleic acid of claim 9, wherein the mammalian NPFF2 receptor is a human NPFF2 receptor.
- 30 11. The nucleic acid of claim 9, wherein the mammalian NPFF2 receptor is a rat NPFF2 receptor.
- 35 12. The nucleic acid of claim 7, wherein the rat NPFF1 receptor has an amino acid sequence identical to that encoded by the plasmid pEXJ-rNPFF1 (ATCC Accession No. 203184).

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13. The nucleic acid of claim 7, wherein the rat NPFF1 receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 2 (SEQ ID NO: 2).
- 5 14. The nucleic acid of claim 8, wherein the human NPFF1 receptor has an amino acid sequence identical to that encoded by plasmid pWE15-hNPFF1 (ATCC Accession No. 203183).
- 10 15. The nucleic acid of claim 8, wherein the human NPFF1 receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 5 SEQ ID NO: 4).
- 15 16. The nucleic acid of claim 8, wherein the human NPFF1 receptor has an amino acid sequence identical to that encoded by plasmid pcDNA3.1-hNPFF1, (ATCC Accession No. 203605).
- 20 17. The nucleic acid of claim 8, wherein the human NPFF1 receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 12 (SEQ ID NO: 8).
- 25 18. The nucleic acid of claim 10, wherein the human NPFF2 receptor has an amino acid sequence identical to that encoded by plasmid pcDNA3.1-hNPFF2b (ATCC Accession No. 203255).
- 30 19. The nucleic acid of claim 10, wherein the human NPFF2 receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 8 (SEQ ID NO: 6).
- 35 20. The nucleic acid of claim 11, wherein the rat NPFF2 receptor has an amino acid sequence identical to that encoded by plasmid pcDNA3.1-rNPFF2-f (ATCC Accession No. Patent Deposit Designation No. PTA-535).

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21. The nucleic acid of claim 11, wherein the rat NPFF2 receptor has an amino acid sequence identical to the amino acid sequence shown in Figures 23A-B (SEQ ID NO: 44).
- 5
22. The nucleic acid of claim 1, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 1 (SEQ ID NO: 1) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence of its complement.
- 10
- 15
23. The nucleic acid of claim 1, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 4 (SEQ ID NO: 3) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.
- 20
- 25
24. The nucleic acid of claim 1, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 7 (SEQ ID NO: 5) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.
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25. The nucleic acid of claim 1, wherein the nucleic acid  
(a) hybridizes to a nucleic acid having the defined  
sequence shown in Figure 11 (SEQ ID NO: 7) under low  
stringency conditions or a sequence complementary  
thereto and (b) is further characterized by its  
ability to cause a change in the pH of a culture of  
CHO cells when a NPFF peptide is added to the culture  
and the CHO cells express the nucleic acid which  
hybridized to the nucleic acid having the defined  
sequence or its complement.
26. The nucleic acid of claim 1, wherein the nucleic acid  
(a) hybridizes to a nucleic acid having the defined  
sequence shown in Figures 22A-C (SEQ ID NO: 43) under  
low stringency conditions or a sequence complementary  
thereto and (b) is further characterized by its  
ability to cause a change in the pH of a culture of  
CHO cells when a NPFF peptide is added to the culture  
and the CHO cells express the nucleic acid which  
hybridized to the nucleic acid having the defined  
sequence or its complement.
27. A purified mammalian NPFF receptor protein.
28. The purified mammalian NPFF receptor protein of claim  
27, wherein the NPFF receptor protein is a NPFF1  
receptor protein.
29. The purified mammalian NPFF receptor protein of claim  
27, wherein the NPFF receptor protein is a NPFF2  
receptor protein.
30. The purified mammalian NPFF1 receptor protein of  
claim 28, wherein the NPFF1 receptor protein is a rat  
NPFF1 receptor protein.
31. The purified mammalian NPFF1 receptor protein of  
claim 28, wherein the NPFF1 receptor protein is a

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human NPFF1 receptor protein.

5 32. The purified mammalian NPFF2 receptor protein of claim 29, wherein the NPFF2 receptor protein is a human NPFF2 receptor protein.

10 33. The purified mammalian NPFF2 receptor protein of claim 29, wherein the NPFF2 receptor protein is a rat NPFF2 receptor protein.

34. A vector comprising the nucleic acid of claim 1.

35. A vector comprising the nucleic acid of claim 6.

15 36. A vector comprising the nucleic acid of claim 9.

37. A vector comprising the nucleic acid of any of claims 22, 23, 24, or 25.

20 38. A vector of any of claims 22, 23, 24, 25, 34, 35, or 36 adapted for expression in a cell which comprises the regulatory elements necessary for expression of the nucleic acid in the cell operatively linked to the nucleic acid encoding the receptor so as to  
25 permit expression thereof, wherein the cell is a bacterial, amphibian, yeast, insect or mammalian cell.

30 39. The vector of claim 38, wherein the vector is a baculovirus.

40. The vector of claim 34, wherein the vector is a plasmid.

35 41. The plasmid of claim 40 designated pEXJ-rNPFF1 (ATCC Accession No. 203184).

42. The plasmid of claim 40 designated pWE15-hNPFF1 (ATCC

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Accession No. 203183).

- 5
43. The plasmid of claim 40 designated pCDNA3.1-hNPFF2b (ATCC Accession No. 203255).
44. The plasmid of claim 40 designated pCDNA3.1-hNPFF1 (ATCC Accession No. 203605).
- 10 45. The plasmid of claim 40 designated pcDNA3.1-rNPFF2-f (ATCC Patent Deposit Designation No. PTA-535).
46. A cell comprising the vector of claim 38.
- 15 47. A cell of claim 46, wherein the cell is a non-mammalian cell.
48. A cell of claim 47, wherein the non-mammalian cell is a Xenopus oocyte cell or a Xenopus melanophore cell.
- 20 49. A cell of claim 46, wherein the cell is a mammalian cell.
50. A mammalian cell of claim 49, wherein the cell is a COS-7 cell, a 293 human embryonic kidney cell, a NIH-3T3 cell, a LM(tk-) cell, a mouse Y1 cell, or a CHO cell.
- 25 51. An insect cell comprising the vector of claim 38.
- 30 52. An insect cell of claim 51, wherein the insect cell is an Sf9 cell, an Sf21 cell or a Trichoplusia ni 5B1-4 cell.
- 35 53. A membrane preparation isolated from the cell of any of claims 46, 47, 49, 50, 51 or 52.
54. A nucleic acid probe comprising at least 15

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- nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within one of the two strands of the nucleic acid encoding the mammalian NPFF1 receptor and contained in plasmid pEXJ-rNPFF1 (ATCC Accession No. 203184), plasmid pWE15-hNPFF1 (ATCC Accession No. 203183), plasmid pCDNA3.1-hNPFF2b (ATCC Accession No. 203255), plasmid pCDNA3.1-hNPFF1 (ATCC Accession No. 203605), or pCDNA3.1-rNPFF2-f (ATCC Patent Deposit Designation No. PTA-535).
55. A nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1 (SEQ ID NO: 1) or (b) the reverse complement thereto.
56. A nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 4 (SEQ ID NO: 3) or (b) the reverse complement thereto.
57. A nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 7 (SEQ ID NO: 5) or (b) the reverse complement thereto.
58. A nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with

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- 5 a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 11 (SEQ ID NO: 7) or (b) the reverse complement thereto.
- 10 59. A nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figures 22A-C (SEQ ID NO: 43) or (b) the reverse complement thereto.
- 15 60. The nucleic acid probe of claim 55, 56, 57, or 58, wherein the nucleic acid is DNA.
- 20 61. The nucleic acid probe of claim 55, 56, 57, or 58, wherein the nucleic acid is RNA.
- 25 62. An antisense oligonucleotide having a sequence capable of specifically hybridizing to the RNA of claim 5, so as to prevent translation of the RNA.
- 30 63. An antisense oligonucleotide having a sequence capable of specifically hybridizing to the genomic DNA of claim 4, so as to prevent transcription of the genomic DNA.
- 35 64. An antisense oligonucleotide of claim 62 or 63, wherein the oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.
65. An antibody capable of binding to a mammalian NPFF receptor encoded by the nucleic acid of claim 1.
66. An antibody of claim 65, wherein the mammalian NPFF receptor is a human NPFF1 receptor.



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67. An antibody of claim 65, wherein the mammalian NPFF receptor is a rat NPFF1 receptor.
- 5 68. An antibody of claim 65, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
69. An antibody of claim 65, wherein the mammalian NPFF receptor is a rat NPFF2 receptor.
- 10 70. An agent capable of competitively inhibiting the binding of the antibody of claim 65 to a mammalian NPFF receptor.
- 15 71. An antibody of claim 65, wherein the antibody is a monoclonal antibody or antisera.
- 20 72. A pharmaceutical composition comprising (a) an amount of the oligonucleotide of claim 62 capable of passing through a cell membrane and effective to reduce expression of a mammalian NPFF receptor and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.
- 25 73. A pharmaceutical composition of claim 72, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.
- 30 74. A pharmaceutical composition of claim 73, wherein the substance which inactivates mRNA is a ribozyme.
- 35 75. A pharmaceutical composition of claim 73, wherein the pharmaceutically acceptable carrier comprises a structure which binds to a mammalian NPFF receptor on a cell capable of being taken up by the cells after binding to the structure.
76. A pharmaceutical composition of claim 75, wherein the pharmaceutically acceptable carrier is capable of

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binding to a mammalian NPFF receptor which is specific for a selected cell type.

- 5 77. A pharmaceutical composition which comprises an amount of the antibody of claim 65 effective to block binding of a ligand to a human NPFF receptor and a pharmaceutically acceptable carrier.
- 10 78. A transgenic, nonhuman mammal expressing DNA encoding a mammalian NPFF receptor of claim 1.
- 15 79. A transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian NPFF receptor.
- 20 80. A transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian NPFF receptor of claim 1 so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian NPFF receptor and which hybridizes to mRNA encoding the mammalian NPFF receptor, thereby reducing its translation.
- 25 81. The transgenic, nonhuman mammal of claim 78 or 79, wherein the DNA encoding the mammalian NPFF receptor additionally comprises an inducible promoter.
- 30 82. The transgenic, nonhuman mammal of claim 78 or 79, wherein the DNA encoding the mammalian NPFF receptor additionally comprises tissue specific regulatory elements.
- 35 83. A transgenic, nonhuman mammal of claim 78, 79, or 80, wherein the transgenic, nonhuman mammal is a mouse.
84. A process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which

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5 comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor.

10 85. A process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting a membrane preparation from cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF  
15 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor.

20 86. The process of claim 84 or 85, wherein the mammalian NPFF receptor is a human NPFF1 receptor.

87. The process of claim 84 or 85, wherein the mammalian NPFF receptor is a human NPFF2 receptor.

25 88. The process of claim 84 or 85, wherein the mammalian NPFF receptor has substantially the same amino acid sequence as the human NPFF1 receptor encoded by plasmid pWE15-hNPFF1 (ATCC Accession No. 203183).

30 89. The process of claim 84 or 85, wherein the mammalian NPFF receptor has substantially the same amino acid sequence as the human NPFF1 receptor encoded by plasmid pCDNA3.1-hNPFF1 (ATCC Accession No. 203605).

35 90. The process of claim 84 or 85, wherein the mammalian NPFF receptor has substantially the same amino acid sequence as the human NPFF2 receptor encoded by plasmid pCDNA3.1-hNPFF2b (ATCC Accession No. 203255).

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91. The process of claim 84 or 85, wherein the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 5 (SEQ ID NO: 4).
- 5 92. The process of claim 84 or 85, wherein the mammalian NPFF receptor has the amino acid sequence shown in Figure 5 (SEQ ID NO: 4).
- 10 93. The process of claim 84 or 85, wherein the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 8 (SEQ ID NO: 6).
- 15 94. The process of claim 84 or 85, wherein the mammalian NPFF receptor has the same amino acid sequence shown in Figure 8 (SEQ ID NO: 6).
- 20 95. The process of claim 84 or 85, wherein the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 12 (SEQ ID NO: 8).
- 25 96. The process of claim 84 or 85, wherein the mammalian NPFF receptor has the same amino acid sequence shown in Figure 12 (SEQ ID NO: 8).
- 27 97. The process of claim 84 or 85, wherein the compound is not previously known to bind to a mammalian NPFF receptor.
- 30 98. A compound identified by the process of claim 97.
- 32 99. A process of claim 84 or 85, wherein the cell is an insect cell.
- 35 100. The process of claim 84 or 85, wherein the cell is a mammalian cell.
101. The process of claim 100, wherein the cell is nonneuronal in origin.

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102. The process of claim 101, wherein the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell.
- 5 103. A process of claim 100, wherein the compound is a compound not previously known to bind to a mammalian NPFF receptor.
- 10 104. A compound identified by the process of claim 103.
- 15 105. A process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both  
20 the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the  
25 chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF  
30 receptor.
- 35 106. A process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting a membrane preparation from cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF

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5 receptor, with both the chemical compound and a  
second chemical compound known to bind to the  
receptor, and with only the second chemical  
compound, under conditions suitable for binding  
of both compounds, and detecting specific binding  
of the chemical compound to the mammalian NPFF  
receptor, a decrease in the binding of the second  
chemical compound to the mammalian NPFF receptor  
10 in the presence of the chemical compound  
indicating that the chemical compound binds to  
the mammalian NPFF receptor.

- 15 107. A process of claim 105 or 106, wherein the  
mammalian NPFF receptor is a human NPFF1  
receptor.
- 20 108. A process of claim 105 or 106, wherein the  
mammalian NPFF receptor is a human NPFF2  
receptor.
- 25 109. The process of claim 105 or 106, wherein the cell  
is an insect cell.
110. The process of claim 105 or 106, wherein the cell  
is a mammalian cell.
111. The process of claim 110, wherein the cell is  
nonneuronal in origin.
- 30 112. The process of claim 111, wherein the nonneuronal  
cell is a COS-7 cell, 293 human embryonic kidney  
cell, a CHO cell, a NIH-3T3 cell, a mouse Y1  
cell, or a LM(tk-) cell.
- 35 113. The process of claim 112, wherein the compound is  
not previously known to bind to a mammalian NPFF  
receptor.

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114. A compound identified by the process of claim 113.

5 115. A method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises

10 (a) contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor;

15 (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor, under conditions permitting binding of compounds known to bind to the mammalian NPFF receptor;

20 (c) determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so

25 (d) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

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116. A method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF

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receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises

- 5 (a) contacting a membrane preparation from cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor under conditions permitting binding of  
10 compounds known to bind to the mammalian NPFF receptor;
- 15 (b) determining whether the binding of a compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds;  
20 and if so
- 25 (c) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.
- 30 117. A method of claim 115 or 116, wherein the mammalian NPFF receptor is a human NPFF1 receptor.
- 35 118. A method of claim 115 or 116, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
119. A method of claim 115 or 116, wherein the cell is a mammalian cell.



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120. A method of claim 119, wherein the mammalian cell is non-neuronal in origin.
- 5 121. The method of claim 120, wherein the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell, or an NIH-3T3 cell.
- 10 122. A method of detecting expression of a mammalian NPFF receptor by detecting the presence of mRNA coding for the mammalian NPFF receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with the nucleic acid probe of any of claims 54, 55, 56, 57, or 58  
15 under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian NPFF receptor by the cell.
- 20 123. A method of detecting the presence of a mammalian NPFF receptor on the surface of a cell which comprises contacting the cell with the antibody of claim 65 under conditions permitting binding of the antibody to the receptor, detecting the  
25 presence of the antibody bound to the cell, and thereby detecting the presence of the mammalian NPFF receptor on the surface of the cell.
- 30 124. A method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a transgenic, nonhuman mammal of claim 81 whose levels of mammalian NPFF receptor activity are varied by use of an inducible promoter which regulates  
35 mammalian NPFF receptor expression.
125. A method of determining the physiological effects of varying levels of activity of mammalian NPFF

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receptors which comprises producing a panel of transgenic, nonhuman mammals of claim 81 each expressing a different amount of mammalian NPFF receptor.

5

126. A method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor comprising administering a compound to the transgenic, nonhuman mammal of claim 78, 81, 82, or 83, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian NPFF receptor, the alleviation of the abnormality, identifying the compound as an antagonist.

10

15

127. The method of claim 126, wherein the mammalian NPFF receptor is a human NPFF1 receptor.

20

128. The method of claim 126, wherein the mammalian NPFF receptor is a human NPFF2 receptor.

129. An antagonist identified by the method of claim 126.

25

130. A pharmaceutical composition comprising an antagonist of claim 129 and a pharmaceutically acceptable carrier.

30

131. A method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of the pharmaceutical composition of claim 130, thereby treating the abnormality.

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132. A method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor comprising administering a compound to the transgenic, nonhuman mammal of claim 78, 81, 82, or 83, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as an agonist.
133. The method of claim 132, wherein the mammalian NPFF receptor is a human NPFF1 receptor.
134. The method of claim 132, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
135. An agonist identified by the method of claim 132.
136. A pharmaceutical composition comprising an agonist identified by the method of claim 135 and a pharmaceutically acceptable carrier.
137. A method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of the pharmaceutical composition of claim 136, thereby treating the abnormality.
138. A method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises:
- (a) obtaining DNA of subjects suffering from the disorder;

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- 5 (b) performing a restriction digest of the DNA with a panel of restriction enzymes;
- (c) electrophoretically separating the resulting DNA fragments on a sizing gel;
- 10 (d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian NPFF receptor and labeled with a detectable marker;
- 15 (e) detecting labeled bands which have hybridized to the DNA encoding a mammalian NPFF1 receptor of claim 1 labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder;
- 20 (f) preparing DNA obtained for diagnosis by steps (a)-(e); and
- 25 (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the
- 30 patterns are the same.

139. The method of claim 138, wherein a disorder associated with the activity of a specific mammalian allele is diagnosed.

35

140. A method of preparing the purified mammalian NPFF receptor of claim 27 which comprises:

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- 5 (a) culturing cells which express the mammalian NPFF receptor;
- (b) recovering the mammalian NPFF receptor from the cells; and
- (c) purifying the mammalian NPFF receptor so recovered.
- 10 141. A method of preparing the purified mammalian NPFF receptor of claim 27 which comprises:
- 15 (a) inserting a nucleic acid encoding the mammalian NPFF receptor into a suitable vector;
- (b) introducing the resulting vector into a suitable host cell;
- 20 (c) placing the resulting cell in suitable condition permitting the production of the mammalian NPFF receptor;
- 25 (d) recovering the mammalian NPFF receptor produced by the resulting cell; and
- (e) isolating and/or purifying the mammalian NPFF receptor so recovered.
- 30 142. A process for determining whether a chemical compound is a mammalian NPFF receptor agonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound under conditions
- 35 permitting the activation of the mammalian NPFF receptor, and detecting an increase in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian

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NPFF receptor agonist.

143. A process for determining whether a chemical compound is a mammalian NPFF receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound in the presence of a known mammalian NPFF receptor agonist, under conditions permitting the activation of the mammalian NPFF receptor, and detecting a decrease in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor antagonist.
144. A process of claim 142 or 143, wherein the mammalian NPFF receptor is a human NPFF1 receptor.
145. A process of claim 142 or 143, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
146. A pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by the process of claim 142 effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.
147. A pharmaceutical composition of claim 146, wherein the mammalian NPFF receptor agonist is not previously known.
148. A pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by the process of claim 143 effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

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149. A pharmaceutical composition of claim 148, wherein the mammalian NPFF receptor antagonist is not previously known.
- 5 150. A process for determining whether a chemical compound specifically binds to and activates a mammalian NPFF receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the  
10 mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the chemical compound under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger  
15 response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the mammalian NPFF receptor.
- 20 151. The process of claim 150, wherein the second messenger response comprises chloride channel activation and the change in second messenger is an increase in the level of inward chloride  
25 current.
152. A process for determining whether a chemical compound specifically binds to and inhibits activation of a mammalian NPFF receptor, which  
30 comprises separately contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical  
35 compound and a second chemical compound known to activate the mammalian NPFF receptor, and with only the second chemical compound, under conditions suitable for activation of the

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5 mammalian NPFF receptor, and measuring the second  
messenger response in the presence of only the  
second chemical compound and in the presence of  
both the second chemical compound and the  
chemical compound, a smaller change in the second  
messenger response in the presence of both the  
chemical compound and the second chemical  
compound than in the presence of only the second  
chemical compound indicating that the chemical  
10 compound inhibits activation of the mammalian  
NPFF receptor.

153. The process of claim 152, wherein the second  
messenger response comprises chloride channel  
15 activation and the change in second messenger  
response is a smaller increase in the level of  
inward chloride current in the presence of both  
the chemical compound and the second chemical  
compound than in the presence of only the second  
chemical compound.  
20

154. A process of any of claims 150, 151, 152, or 153,  
wherein the mammalian NPFF receptor is a human  
NPFF1 receptor.  
25

155. A process of any of claims 150, 151, 152, or 153,  
wherein the mammalian NPFF receptor is a human  
NPFF2 receptor.

30 156. The process of any of claims 150, 151, 152, or  
153, wherein the cell is an insect cell.

157. The process of any of claims 150, 151, 152, or  
153, wherein the cell is a mammalian cell.  
35

158. The process of claim 157, wherein the mammalian  
cell is nonneuronal in origin.



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159. The process of claim 158, wherein the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.
- 5 160. The process of claim 150, 151, 152, or 153, wherein the compound is not previously known to bind to a mammalian NPFF receptor.
- 10 161. A compound determined by the process of claim 160.
- 15 162. A pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by the process of claim 150 or 151 effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.
- 20 163. A pharmaceutical composition of claim 162, wherein the mammalian NPFF receptor agonist is not previously known.
- 25 164. A pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by the process of claim 152 or 153 effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.
- 30 165. A pharmaceutical composition of claim 164, wherein the mammalian NPFF receptor antagonist is not previously known.
- 35 166. A method of screening a plurality of chemical compounds not known to activate a mammalian NPFF receptor to identify a compound which activates the mammalian NPFF receptor which comprises:

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- 5 (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds not known to activate the mammalian NPFF receptor, under conditions permitting activation of the mammalian NPFF receptor;
- 10 (b) determining whether the activity of the mammalian NPFF receptor is increased in the presence of the compounds; and if so
- 15 (c) separately determining whether the activation of the mammalian NPFF receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound which activates the mammalian NPFF receptor.
- 20 167. A method of claim 166, wherein the mammalian NPFF receptor is a human NPFF1 receptor.
- 25 168. A method of claim 166, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
- 30 169. A method of screening a plurality of chemical compounds not known to inhibit the activation of a mammalian NPFF receptor to identify a compound which inhibits the activation of the mammalian NPFF receptor, which comprises:
- 35 (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds in the presence of a known mammalian NPFF receptor agonist, under conditions permitting activation of the mammalian NPFF receptor;
- (b) determining whether the activation of the

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mammalian NPFF receptor is reduced in the presence of the plurality of compounds, relative to the activation of the mammalian NPFF receptor in the absence of the plurality of compounds; and if so

5

(c) separately determining the inhibition of activation of the mammalian NPFF receptor for each compound included in the plurality of compounds, so as to thereby identify the compound which inhibits the activation of the mammalian NPFF receptor.

10

170. A method of claim 169, wherein the mammalian NPFF receptor is a human NPFF1 receptor.

15

171. A method of claim 169, wherein the mammalian NPFF receptor is a human NPFF2 receptor.

20

172. A method of any of claims 166, 167, 168, 169, 162, or 163, wherein the cell is a mammalian cell.

173. A method of claim 172, wherein the mammalian cell is non-neuronal in origin.

25

174. The method of claim 173, wherein the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

30

175. A pharmaceutical composition comprising a compound identified by the method of claim 166 or 167 effective to increase mammalian NPFF receptor activity and a pharmaceutically acceptable carrier.

35

176. A pharmaceutical composition comprising a compound identified by the method of claim 169 or

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170 effective to decrease mammalian NPFF receptor activity and a pharmaceutically acceptable carrier.

- 5        177.     A method of treating an abnormality in a subject  
             wherein the abnormality is alleviated by  
             increasing the activity of a mammalian NPFF  
             receptor which comprises administering to the  
10        subject an amount of a compound which is a  
             mammalian NPFF receptor agonist effective to  
             treat the abnormality.
178.     A method of claim 177, wherein the abnormality is  
             a lower urinary tract disorder, a regulation of  
15        a steroid hormone disorder, an epinephrine  
             release disorder, a gastrointestinal disorder,  
             irritable bowel syndrome, a cardiovascular  
             disorder, an electrolyte balance disorder,  
             diuresis, hypertension, hypotension, diabetes,  
20        hypoglycemia, a respiratory disorder, asthma, a  
             reproductive function disorder, an immune  
             disorder, an endocrine disorder, a  
             musculoskeletal disorder, a neuroendocrine  
             disorder, a cognitive disorder, a memory  
25        disorder, a sensory modulation and transmission  
             disorder, a motor coordination disorder, a  
             sensory integration disorder, a motor integration  
             disorder, a dopaminergic function disorder, a  
             serotonergic function disorder, an appetite  
30        disorder, obesity, a sensory transmission  
             disorder, an olfaction disorder, a sympathetic  
             innervation disorder, pain, psychotic behavior,  
             morphine tolerance, nicotine addiction, opiate  
             addiction, affective disorder, or migraine.
- 35        179.     A method of treating an abnormality in a subject  
             wherein the abnormality is alleviated by  
             decreasing the activity of a mammalian NPFF

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receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor antagonist effective to treat the abnormality.

5

180. A method of claim 179, wherein the abnormality is a lower urinary tract disorder, a regulation of steroid hormone disorder, an epinephrine release disorder, a gastrointestinal disorder, irritable bowel syndrome, a cardiovascular disorder, an electrolyte balance disorder, diuresis, hypertension, hypotension, diabetes, hypoglycemia, a respiratory disorder, asthma, a reproductive function disorder, an immune disorder, an endocrine disorder, a musculoskeletal disorder, a neuroendocrine disorder, a cognitive disorder, a memory disorder, a sensory modulation and transmission disorder, a motor coordination disorder, a sensory integration disorder, a motor integration disorder, a dopaminergic function disorder, a serotonergic function disorder, an appetite disorder, obesity, a sensory transmission disorder, an olfaction disorder, a sympathetic innervation disorder, pain, psychotic behavior, morphine tolerance, nicotine addiction, opiate addiction, affective disorder, or migraine.

181. A process for making a composition of matter which specifically binds to a mammalian NPFF receptor which comprises identifying a chemical compound using the process of any of claims 84, 85, 105, 106, 115, or 116 and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

182. A process for making a composition of matter which specifically binds to a mammalian NPFF

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receptor which comprises identifying a chemical compound using the process of any of claims 134, 150, or 166 and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

- 5
183. A process for making a composition of matter which specifically binds to a mammalian NPFF receptor which comprises identifying a chemical compound using the process of any of claims 143, 152, 169 and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.
- 10
184. The process of any of claims 181, 182, or 183, wherein the mammalian NPFF receptor is a human NPFF1 receptor.
- 15
185. The process of any of claims 181, 182, or 183, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
- 20
186. A process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by the process of any of claims 84, 85, 105, 106, 115, or 116 or a novel structural and functional analog or homolog thereof.
- 25
187. A process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by the process of any of claims 142, 150, or 166 or a novel structural and functional analog or homolog thereof.
- 30
- 35

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- 5 188. A process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by the process of any of claims 143, 152, or 169 or a novel structural and functional analog or homolog thereof.
- 10 189. The process of any of claims 186, 187, or 188, wherein the mammalian NPFF receptor is a human NPFF1 receptor.
- 15 190. The process of any of claims 186, 187, or 188, wherein the mammalian NPFF receptor is a human NPFF2 receptor.
- 20 191. A method of claim 117, wherein the cell is a mammalian cell.
- 25 192. The method of claim 191, wherein the mammalian cell is non-neuronal in origin.
193. The method of claim 192, wherein the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell, or an NIH-3T3 cell.
- 30 194. A method of claim 118, wherein the cell is a mammalian cell.
195. The method of claim 194, wherein the mammalian cell is non-neuronal in origin.
- 35 196. The method of claim 195, wherein the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell, or an NIH-3T3 cell.

Figure 1

1 ACCCTTCCTGGGCCCCAGTCTACCCGGCTTGAGGTTGCCCGGCTCCTTTGGAGAGTGTCCC  
61 GGAGCAGACAGTATGGAGGGGAGCCCTCCGAGCCCTCCCAACGGGAGCTGGCCCTGGGT  
121 CAGAACGGGAGTGTGTGGAGACAGGATGGCAACCGGCTCAGCTTCTCTCTCTACTAC  
181 CAACACTCCTCTCCGGTGGCAGCCATGTTCTACGCGGCTACGTGCTCATCTTCTCTCTC  
241 TGCATGGTGGCAACACCCCTGGTCTGCTTCTATGTTGCTCAAGAACGGGACATGGGCACT  
301 GTCACCAACATGTTTATCCTCAACCTGGCCGTACGCGACCTGCTGGTGGGCATCTTCTGC  
361 ATGCCACACACCCCTGTGGACACCTTATCACTGTTGGCTTTTGACAAACGCCACATGC  
421 AAGATGAGCGGCTTGGTGAGGGCATGTCCGTGTCTGCATCGGTTTACACTGGTGGCC  
481 ATCGCTGTGGAAGGTTCCGCTGCATCGTGCAACCTTCCCGGAGAGCTGACCTTCGG  
541 AAGCGCTGTTCAACCATCGCGGTGATCTGGGCTCTGGCGCTGCTCATCATGTGCCCTCG  
601 GCGGTCACTCTGACAGTCAACCGAGAGGAGCACTTCACTTCATGCTGGATGCTCGTAACCGC  
661 TCCTACCCGCTCTACTCGTGTGGAGGGCTGCCCCGAGAGGGCATGGCAGGTCTAC  
721 ACCGGGTGCTCTTCGGCACATCTACCTGGTGCCGCTGGGCTCATCGTAGTGATGAC  
781 GTGGCATCGCGCGCAGCTATGCCAGGCCCGCGGTCTGCGCGGACACGGAGGAGCGG  
841 GTGCCGAGGGTGGCCGCACTTCGCGCGGTAGGGCCCGCTGGTGGTGCACATGCTGGTCATG  
901 GTGGCGCTCTTCTTACGTTGTCTGGTGGCTGCACTGCACTGCTGCGCTTCCCTTTGGCA  
961 TATGGGAGCTGAGCGAGCTGCACTGCACTGCTGCGGTCTACGGCTTCCCTTTGGCA  
1021 CACTGGCTGGCTTCTTCCACAGAGCGGCCCAACCCCATCATCTACGGCTACTTCAACGAG  
1081 PACTTCGCGCGGCTTCCAGGCTGCCCTCCGTGCAACAGCTCTGCTGGCTTCCCTGGGCTG  
1141 GCCACAAAGCAAGCCTACTCGGAGCGGCCCAACCGCTCTGCGGAGGCGGCTGGTGGTG  
1201 GACGTGCAACCCAGCGACTCCGGCTGCCATCAGAGTCTGGCCCAAGCAACGGGTCCCA  
1261 GGGCTGGCCGGCTGCCACTGGGCAATGGGCTGTGGCCCATCAGGATGGCCCGGGGAA  
1321 GGGCCAGGCTGCACACCATGCCCTCACCATCCCGGCTGGACATTTGAGGTGGTCCA  
1381 GAGAAGGGAGGGCCAGTAGTCTGTGGCCC



Figure 2

1	M E A E P S Q P P N G S W P L G Q N G S	20
21	D V E T S M A T S L T F S S Y Y Q H S S	40
41	P V A A M F I A A Y V L I F L L C M V G	60
61	N T L V C F I V L K N R H M R T V T N M	80
81	F I L N L A V S D L L V G I F C M P T T	100
101	L V D N L I T G W P F D N A T C K M S G	120
121	L V Q G M S V S A S V F T L V A I A V E	140
141	R F R C I V H P F R E K L T L R K A L F	160
161	T I A V I W A L A L L I M C P S A V T L	180
181	T V T R E E H H F M L D A R N R S Y P L	200
201	Y S C W E A W P E K G M R K V Y T A V L	220
221	F A H I Y L V P L A L I V V M Y V R I A	240
241	R K L C Q A P G F A R D T E E A V A E G	260
261	G R T S R R R A R V V H M L V M V A L F	280
281	F T L S W L P L W V L L L L I D Y G E L	300
301	S E L Q L H L L S V Y A F P L A H W L A	320
321	F F H S S A N P I I Y G Y F N E N F R R	340
341	G F Q A A F R A Q L C W P P W A A H K Q	360
361	A Y S E R P N R L L R R R V V V D V Q P	380
381	S D S G L P S E S G P S S G V P G P G R	400
401	L P L R N G R V A H Q D G P G E G P G C	420
421	N H M P L T I P A W N I	432

Figure 3

1 M E A E P S Q P P N G C W P L G Q N G C 20  
21 D V E T S M A T S L T F S S Y Y Q H S S 40  
41 P V A A M F I A A Y V L I F L L C M V C 60  
61 N T L V C F I V L K N R H M R T V T N M 80  
81 F I L N L A V S D L L V G I F C M P T T 100  
101 L V D N L I T G W P F D N A T C K M S G 120  
121 L V O G M S V S A S V F T L V A I A V E 140  
141 R F R C I V H P F R E K L T L R K A L F 160  
161 T I A V I W A L A L L I M C P S A V T L 180  
181 T V T R E E H H F M L D A R N R S Y P L 200  
201 Y S C W E A W P E K G M R K V Y T A V L 220  
221 F A H I Y L V P L A L I V V M Y V R I A 240  
241 R K L C Q A P G P A R D T E E A V A E G 260  
261 G R T S R R R A R V V H M L V M V A L F 280  
281 F T L S W L P L W V L L L L I D Y G E L 300  
301 S E L Q L H L L S V Y A F P L A H W L A 320  
321 F F H S S A N P I I Y G Y F N E N E R R 340  
341 G F Q A A F R A Q L C W P P W A A H K Q 360  
361 A Y S E R P N R L L R R R V V V D V Q P 380  
381 S D S G L P S E S G P S S G V P G P G R 400  
401 L P L R N G R V A H Q D G P G E G P G C 420  
421 N H M P L T I P A W N I 432

Figure 4

```
1  GAGCCCTCCAGCCTCCCAACAGCAGTTGGCCCCCTTAGTCAGAAATGGGACTAACACTGAG    60
61  GCCACCCCGGCTACAAACCTCACCTTCTCCTCCTACTATCAGCACACCTCCCCCTGTGGCG    120
121  GCCATGTTTCATTGTGGCCTATGCGCTCATCTTCCTGCTCTGCATGTTGGGCAACACCCCTG    180
181  GTCTGTTTCATCGTGCTCAA                                         200
```

Figure 5

1	E	P	S	Q	P	P	N	S	S	W	P	L	S	Q	N	G	T	N	T	E	20
21	A	T	P	A	T	N	L	T	F	S	S	Y	Y	Q	H	T	S	P	V	A	40
41	A	M	F	I	V	A	Y	A	L	I	F	L	L	C	M	V	G	N	T	L	60
61	V	C	F	I	V	L														66	

Figure 6

```
1 MEAEPSPNGSWPLGQNGSDVETSMATSLTSSYYQHSSPVAA1MEIAAY rNPFF1
  ||||| |||| |||| | . ||. ||||| ||||| ||||| ||
1 ...EPSQPPNSSWPLSQNGTNT1ATPATNLTSSYYQHTSPVAA1MEIVAY hNPFF1

51 VLIFLLCMVGNTLVCFIVL rNPFF1
  ||||| ||||| ||||| |||||
48 ALIFLLCMVGNTLVCFIVL hNPFF1
```

Figure 7

1 GCGACAGGGCTGCGCCGGAGAGGTTTCATCATGAAATGAGAAATGGGACACAACTCTTCA  
61 GAFACTGGCATCCCATCTGGAATGTCAATGACACAAAGCATCATCTGTACTCAGATATT  
121 AATATTACCTATGTGAACACTACTTCTTACCCAGGCTCAAGTGGCAGCAATCTTCATTATT  
181 TCCTACTTTCTGATCTTCTTTTGTGCATGATGGGAAATACTGTGTTTTCCTTTATTGTA  
241 ATGAGGAACAACATATGCACACAGTCACATACTCTTTCATCTTAAACCTGGCCATAAGT  
301 GATTACTAGTTGGCATATTCTGCATGCCCTATAACACTGCTGGACAATATTATAGCAGGA  
361 TGGCCATTTGGAACACAGATGTGCAAGATCAGTGGATTGGTCCAGGGAATATCTGTCGCA  
421 GCTTCAGTCTTTACGTTAGTTGCAATTGCTGTAGATAGGTTCCAGTGTGTGGTCTACCCCT  
481 TTTAAACCAAGCTCACTATCAAGACAGCGTTTGTCTATTATATGATCATCTGGGTCCTA  
541 GCCATCACCAATTATGTCTCCATCTGCAGTAATGTTACATGTGCAAGAGAAATAATTAC  
601 CGAGTGAGACTCAACTCCAGATAAACCAGTCCAGTCTACTGGTCCGGGAAGACTGG  
661 CCAATCAGGAATGAGGAAGATCTACACCAGTGTGTGTTGCCAACATCTACCTGGCT  
721 CCCCTCTCCCTCATTTGTCATCATGTATGGAAGGATTGGAATTTCACTCTTCAGGGCTGCA  
781 GTTCTCACACAGGAGGAAGAACCCAGGAGCAGTGGCAGCGTGTGTCAGGAAGAGCAG  
841 AAGATCAATTAAGATGCTCTCAGACTACGCTGACCTTTCTCCAAATGAACAGCATCATC  
901 TGGACTCTAATGATGCTCTCAGACTACGCTGACCTTTCTCCAAATGAACAGCATCATC  
961 AACATCTACATCTACCCCTTTTGCACACACTGGCTGGCATTCGGCAACAGCAGTGTCAATCCC  
1021 ATCATTATGGTTTCTTCAACGAGCAATTCGCGCGTGGTTTCCAAAGAGCTTCCAGCTC  
1081 CAGCTCTGCCAATAAAGAGCAAGGCTATGGAAGCTTATGCCCTAAAGCTAAAGCCAT  
1141 GTGCTCATAAACACATCTAATCAGCTTGTCCAGGATCTACATTTCAAAACCTCATGGG  
1201 GAAACCTTGCTTTATAGGAAGAGTGCTGAACCCCAACAGGAATTAGTGTGGAGAA  
1261 TTAAAGAAACTACTAACAGCAGTGAGATTAAAGAGCTA

Figure 8

1	M N E K W D T N S S E N W H P I W N V N	20
21	D T K H H L Y S D I N I T Y V N Y Y L H	40
41	Q P Q V A A I F I I S Y F L I F F L C M	60
61	M G N T V V C F I V M R N K H M H T V T	80
81	N L F I L N L A I S D L L V G I F C M P	100
101	I T L L D N I I A G W P F G N T M C K I	120
121	S G L V Q G I S V A A S V F T L V A I A	140
141	V D R F Q C V V Y P F K P K L T I K T A	160
161	F V I I M I I W V L A I T I M S P S A V	180
181	M L H V Q E E K Y Y R V R L N S Q N K T	200
201	S P V Y W C R E D W P N Q E M R K I Y T	220
221	T V L F A N I Y L A P L S L I V I M Y G	240
241	R I G I S L F R A A V P H T G R K N Q E	260
261	Q W H V V S R K K Q K I I K M L L I V A	280
281	L L F I L S W L P L W T L M M L S D Y A	300
301	D L S P N E I O I I N I Y I Y P F A H W	320
321	L A F G N S S V N P I I Y G F F N E N F	340
341	R R G F Q E A F Q L Q L C Q K R A K P M	360
361	E A Y A L K A K S H V L I N T S N Q L V	380
381	Q E S T F Q N P H G E T L L Y R K S A E	400
401	K P Q Q E L V M E E L K E T T N S S E I	420

Figure 9

1 M N E K W D T N S S E N W H P I W N V N 20  
21 D T K H H L Y S D I N I T Y V N Y Y L H 40  
41 Q P Q V A A I F I I S Y F L I F F L C M 60  
61 M G N T V V C F I V M R N K H M H T V T 80  
81 N L F I L N L A I S D L L V G I F C M P 100  
101 I T L L D N I I A G W P F G N T M C K I 120  
121 S G L V O G I S V A A S V F T L V A I A 140  
141 V D R F Q C V V Y P F K P K L T I K T A 160  
161 F V I I M I I W V L A I T I M S P S A V 180  
181 M L H V Q E E K Y Y R V R L N S Q N K T 200  
201 S P V Y W C R E D W P N Q E M R K I Y T 220  
221 T V L F A N I Y L A P L S L I V I M Y G 240  
241 R I G I S L F R A A V P H T G R K N Q E 260  
261 Q W H V V S R K K Q K I I K M L L I V A 280  
281 L L F I L S W L P L W T L M M L S D Y A 300  
301 D L S P N E L Q I I N I Y I Y P F A H W 320  
321 L A F G N S S V N P I I Y G F F N E N F 340  
341 R R G F Q E A F Q L Q L C Q K R A K P M 360  
361 E A Y A L K A K S H V L I N T S N Q L V 380  
381 Q E S T F Q N P H G E T L L Y R K S A E 400  
401 K P Q Q E L V M E E L K E T T N S S E I 420



Figure 10

```

rNPFF1 MEAEPSQPPNGSWPLGQNGSDVETSMAT..SLTFSSYYQHSSPVAAMFIA 48
      | . . . | . . . : . . : | | | | |
hNPFF2 MNEKWDTNSSSENWHDIWNVNDTKHHLYS DINITYVNYLHQPQVA AIFII 50

rNPFF1 AYVLIFLLCMVGNTLVCFIVLKNRHMRTVTNMFILNLAVSDLLVGIFC MP 98
      . | | | | | . | | | | | : | | | | | : | | | | |
hNPFF2 SYFLIFFLCMMGNTVVC FIVMRNKHMH TVTNLFILNL AISDLLVGIFC MP 100

rNPFF1 TTLVDNLITGWPF DNATCKMSG LVOGMSVSASVETLV AIAVERFCIVHP 148
      | | | | | : | | | | | | | | | | | | | | | | | | | | |
hNPFF2 TTLVDNIIAGWPF DNATCKMSG LVOGMSVSASVETLV AIAVERFCIVHP 148

rNPFF1 FREKLT LRKALFTI A VIWALALLIMCPSAVTLTVTREEHH.FMLDARNRS 197
      | : | | | : | | | | | : | | | | | | | | | | | | | | |
hNPFF2 FKPKLT IKTAFVI IMI IWVLAITIMSPSAVMLHVQEEKY YRVLNSQNKT 200

rNPFF1 YPLYSCWEAWPEKGMRKVYTAVLFAHIYLVPLALIVVMYVRIARKLCQAP 247
      | . | | | | | . | | | | | | | | | | | | | | | | | | | |
hNPFF2 SPVYWCRE DWPNQEMRKIYTTVLFANIY LAPLSLIVIMYGRIGISLFRAA 250

rNPFF1 GPARDTEEAVAEGGRTSRRRARVVHMLVMVALFFTL SWLPLWVLLLLIDY 297
      | . . . : | | | : | | | | | | | | | | | | | | | | |
hNPFF2 VPHTGRKNQ.EQWHVVSRRKKQKI I KMLLIVALLFILSWLPLWTLMMLS DY 299

rNPFF1 GELSELQLHLLSVYAFPLAHWLAFFHSSANPIIYG YFNENFRRGFQAAFR 347
      : | | : | : : : | | | | | | | | | | | | | | | | | |
hNPFF2 ADLSPNELQIINIYIYPFAHWLAFGNSSVNP I IYGFFNENFRRGFQEA FQ 349

rNPFF1 AQLCWPPWAAHKQAYSERPNRLLRRRVVVDVQPSDSGLP.SESGPSSGVP 396
      | | | . . . . | | | | | | | | | | | | | | | | | |
hNPFF2 LQLCQKRAKPM EAYALKAKSHVLINTSNQLVQESTFQNP HGETLLYRKSA 399

rNPFF1 GPGRLPLRNGRVAHQDGPGE GPGCNHMLPTIPAWNI 432
      | . . . | | | | | | | | | | | | | | | | | | | |
hNPFF2 EKPQQELVMEELKET TNSSEI..... 420

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Figure 11

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1  ATGAGGGGAGCCCTCCAGCCTCCCAACAGCAGTTGGCCCCAAGTCAGAATGGGACT 60
61  AACACTGAGGCCACCCCGGTACAAACCTCACCTTCTCCTCCTACTATCAGCACACCTCC 120
121  CCTGTGGGGCCATGTTTCAATTGTGGCCTATGGCTCATCTTCTGCTCTGCATGGTGGGC 180
181  AACACCCTGGTCTGTTTCAATCGTGTCAAGAACCGGCACATGCATACTGTCAACCAACATG 240
241  TTCAATCCTCAACCTGGCTGTCAAGTACCTGCTGGTGGGCACTTCTGTCATGCCACCAACC 300
301  CTTGTGGACAACTCATCACTGGGTGGCCCTTCGACAAATGCCACATGCAAGATGAGCGGC 360
361  TTGGTGCAGGGCATGTCTGTGTGGCTTCCGTTTTCACACTGGTGGCCATTGCTGTGGAA 420
421  AGGTTCCGCTGCATCGTGCACCCCTTTCGGCGAGAAGCTGACCCCTGCGGAAGGCGCTCGTC 480
481  ACCATCGCCGTCATCTGGGCCCTGGCGCTGCTCATCATGTGTCCCTCGGCCGTCACGCTG 540
541  ACCGTACCCGTGAGGAGCACCACTTCAATGTTGGAGCGCCCTCACCGCTCCTACCCCTCTC 600
601  TACTCCTGCTGGGAGGCCCTGGCCCCGAGAAGGCGATGCGCAAGGCTCTACACCACATGTGCTC 660
661  TTCTCGCACATCTACCTGGGCGCGCTGGCGCTCATCGTGTGTCATGTACGCCCGCATCGCG 720
721  CGCAAGCTCTGCCAGGCCCGGGCCCCGGGGCGGAGGAGGCTGCGGACCCCGGA 780
781  GCATCGCGGGCGAGAGCGCGGTGGTGCACATGCTGGTCAATGGTGGCGCTGTTCTTCACG 840
841  CTGTCTGCTGGCTGCCGCTCTGGGCGCTGCTGCTGCTCATCGACTACGGGCGAGCTCAGCGCG 900
901  CCGCAGCTGCACCTGGTCAACCTTACGCTTCCCCCTTCCGCGCACTGGCTGGCCCTTCTTC 960
961  AACAGCAGCGCCAAACCCCATCATCTACGGCTACTTCAACGAGAACTTCCGCCCGCGGCTTC 1020
1021  CAGGCCGCTTCCGCGCCCGCTCTGCCCGCGCCCGTGGGGAGCCACAAAGAGGCTTAC 1080
1081  TCCGAGCGGCCCGGGGCTTCTGCACAGCGGGTCTTCTGTTGTTGGTGGGCCCCAGCGAC 1140
1141  TCCGGGCTGCCCTCTGAGTCGGGCCCTAGCACTGGGGCCCCCAGGCCCGCCGCTCCCG 1200
1201  CTGCGGAATGGCGGGTGGCTCACCACGGCTTGCCACAGGAGGAGGCTGGCTGCTCCAC 1260
1261  CTGCCCCCTCACCATTCAGCCCTGGGATATCIGA 1293

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Figure 12

1	M E G E P S Q P P N S S W P L S Q N G T	20
21	N T E A T P A T N L T F S S Y Y Q H T S	40
41	P V A A M F I V A Y A L I F L L C M V G	60
61	N T L V C F I V L K N R H M H T V T N M	80
81	F I L N L A V S D L L V G I F C M P T T	100
101	L V D N L I T G W P F D N A T C K M S G	120
121	L V Q G M S V S A S V F T L V A I A V E	140
141	R F R C I V H P F R E K L T L R K A L V	160
161	T I A V I W A L A L L I M C P S A V T L	180
181	T V T R E E H H F M V D A R N R S Y P L	200
201	Y S C W E A W P E K G M R R V Y T T V L	220
221	F S H I Y L A P L A L I V V M Y A R I A	240
241	R K L C Q A P G P A P G G E E A A D P R	260
261	A S R R R A R V V H M L V M V A L F F T	280
281	L S W L P L W A L L L L I D Y G Q L S A	300
301	P Q L H L V T V Y A F P F A H W L A F F	320
321	N S S A N P I I Y G Y F N E N F R R G F	340
341	Q A A F R A R L C P R P S G S H K E A Y	360
361	S E R P G G L L H R R V F V V V R P S D	380
381	S G L P S E S G P S S G A P R P G R L P	400
401	L R N G R V A H H G L P R E G P G C S H	420
421	L P L T I P A W D I	430

Figure 13

1 M E G E P S Q P P N S S W P L S Q N G T 20  
21 N T E A T P A T N L T F S S Y Y Q H T S 40  
41 P V A A M F I V A Y A L I F L L C M V G 60  
61 N T L V C F I V L K N R H M H T V T N M 80  
81 F I L N L A V S D L L V G I F C M P T T 100  
101 L V D N L I T G W P F D N A T C K M S G 120  
121 L V Q G M S V S A S V F T L V A I A V E 140  
141 R F R C I V H P F R E K L T L R K A L V 160  
161 T I A V I W A L A L L I M C P S A V T L 180  
181 T V T R E E H H F M V D A R N R S Y P L 200  
201 Y S C W E A W P E K G M R R V Y T T V L 220  
221 F S H I Y L A P L A L I V V M Y A R I A 240  
241 R K L C Q A P G P A P G G E E A A D P R 260  
261 A S R R R A R V V H M L V M V A L F F T 280  
281 L S W L P L W A L L L L I D Y G Q L S A 300  
301 P Q L H L V T V Y A F P F A H W L A F F 320  
321 N S S A N P I I Y G Y F N E N F R R G F 340  
341 Q A A F R A R L C P R P S G S H K E A Y 360  
361 S E R P G G L L H R R V F V V V R P S D 380  
381 S G L P S E S G P S S G A P R P G R L P 400  
401 L R N G R V A H H G L P R E G P G C S H 420  
421 L P L T I P A W D I 430

hNPF2	1	MNEKWDNTSSSENWHPIWNVDNTHKHLYSIDINITYVNYLHQPVAAIFII	50
hNPF1	1	..MEGEPSQPPNSSWPLSQNGTNTTEATPATNLTFSSYYQHTSPVAAMFIV	48
hNPF2	51	SYFLIFFLCMMGNTVVCFIVMRNKHMHMTVTNLFILNLAISDLLVGIFCMP	100
hNPF1	49	AYALIFLLCMVGNTLVCFIVLKNRHMHTVTNMFILNLAVSDLLVGIFCMP	98
hNPF2	101	ITLLDNIIAGWPFGNTMCKISGLVQGISVAASVFTLVAIAVDRFQCVVYP	150
hNPF1	99	TTLVDNLITGWPFDNATCKMSGVLVQGMVSASVFTLVAIAVERFRFCIVHP	148
hNPF2	151	FKPKLTIKTAFTVIIMIIWVLAITIMSPSAVMLHVQEEKYYRVRLNSQNK	200
hNPF1	149	FREKLTLRKALVTIAVIWALALLIMCPSAVTLTVTREEHH.FMVDARNRS	197
hNPF2	201	SPVYWCREDDWPNQEMRKIYTTVLFANIYLAFLSLIVIMYGRIGISLFRAA	250
hNPF1	198	YPLYSCWEAWPEKGMRRVYTTVLFSHIYLAFLALIVVMYARIARKLCQAP	247
hNPF2	251	VPHTGRKNQEQWHVVSRRKKQKIIKMLLIVALLFILSWLPLWTIMMLSDYA	300
hNPF1	248	GPAPGGEEAADPR.ASRRRARVVHMLVMVALFFTLWLPLWALLLLIDYG	296
hNPF2	301	DLSPNELQIINIYIYPFAHWLAFFGNSSVNPIIYGFFNENFRRGFQEAQFL	350
hNPF1	297	QLSAPQLHLVTVYAFPPFAHWLAFFNSSANPIIYGFFNENFRRGFQAAFA	346
hNPF2	351	QLCQKRAKPMEAYALKAKSHVLINTSNQLVQESTFQNPHEGTLTYRKS	400
hNPF1	347	RLC.PRPSGSHKEAYSERPGGLLHRRVFVVRPSDGLPSESGPSSGAPR	395
hNPF2	401	KPQQELVMEELKETTNSSEI*.....	420
hNPF1	396	PGRLPLRNGRVAHHGLPREGPGCSHLPLTIPAWDI*	431

Figure 15A

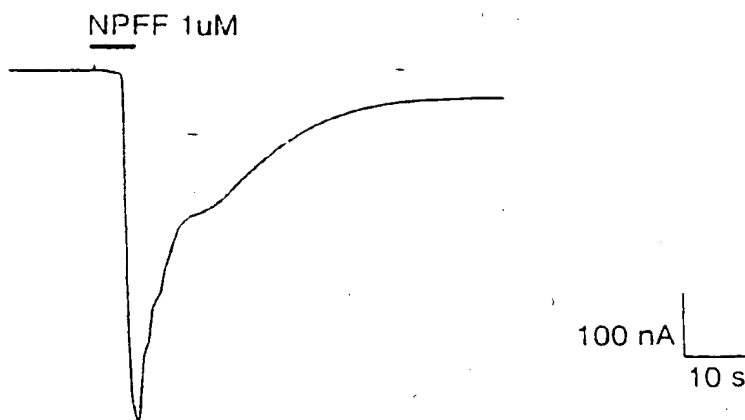


Figure 15B

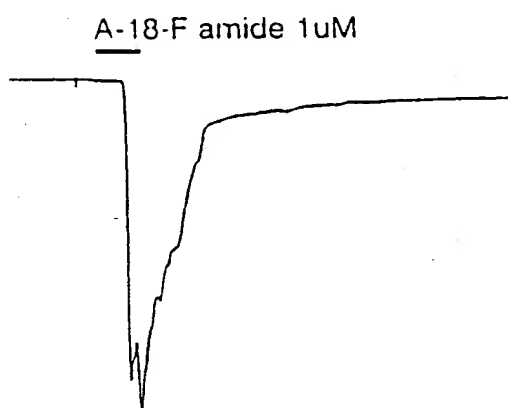


Figure 15C

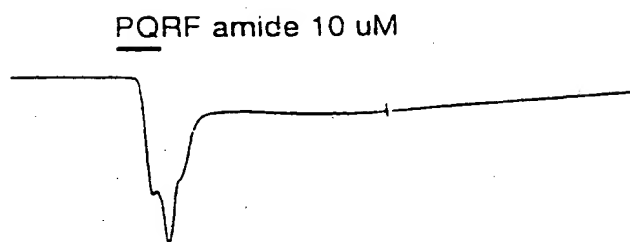


Figure 16A

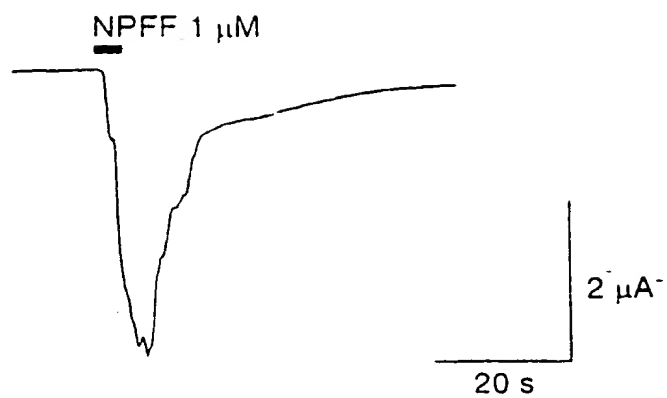


Figure 16B



Figure 16C

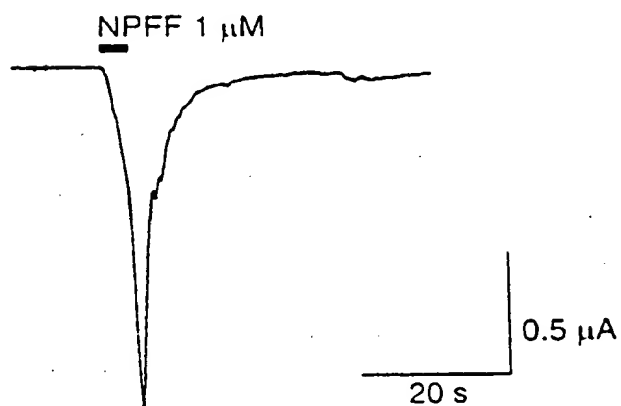


Figure 17A

Microphysiometer:  
Snorf2 (+/- G<sub>o</sub>/G<sub>z</sub>) in CHO

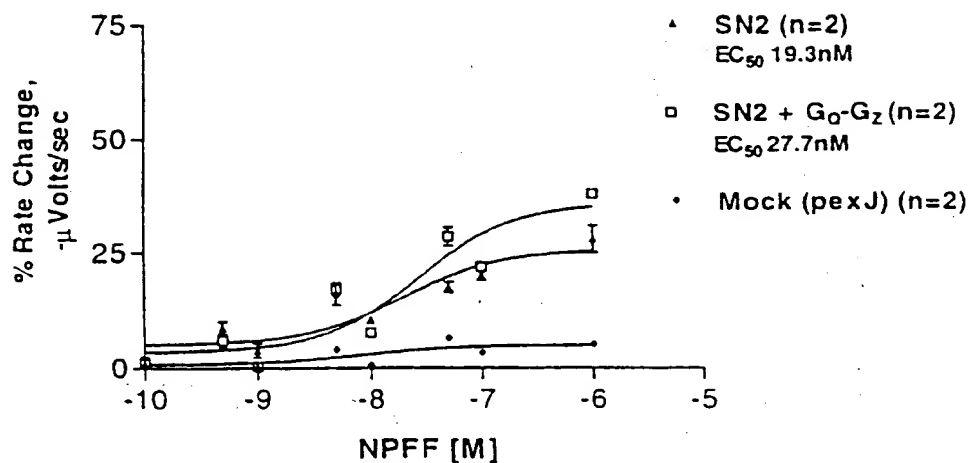
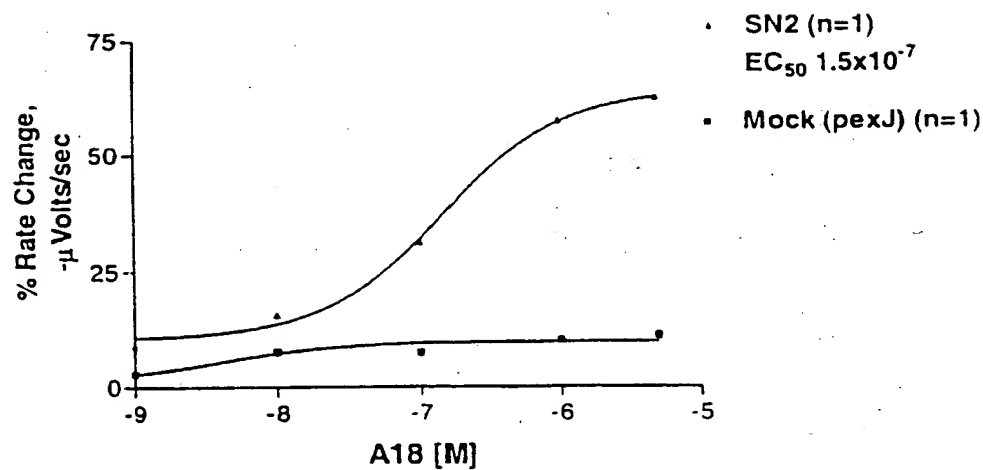


Figure 17B

Microphysiometer:  
Snorf2 in CHO





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Figure 18A

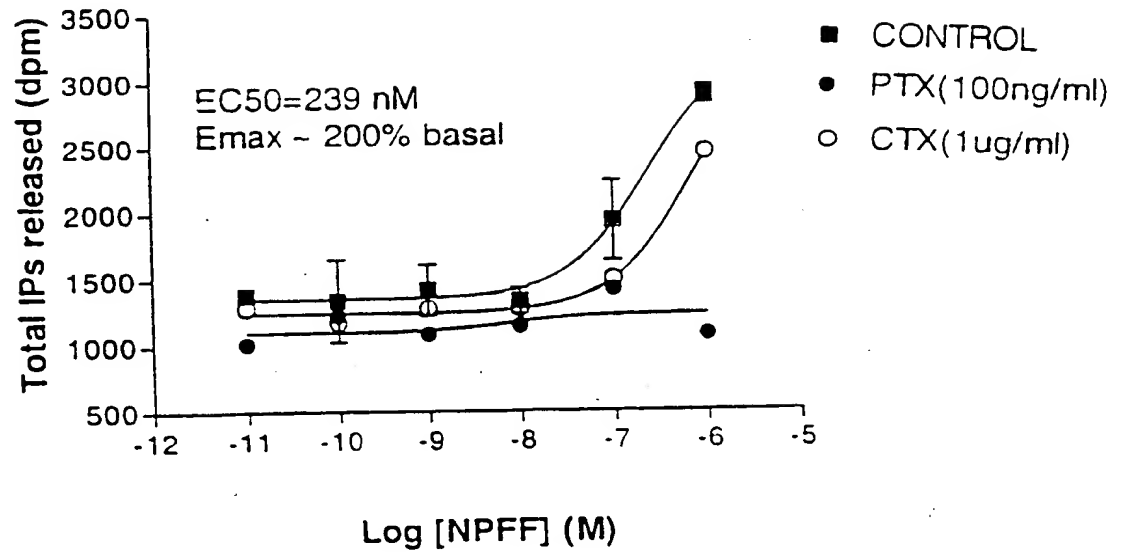
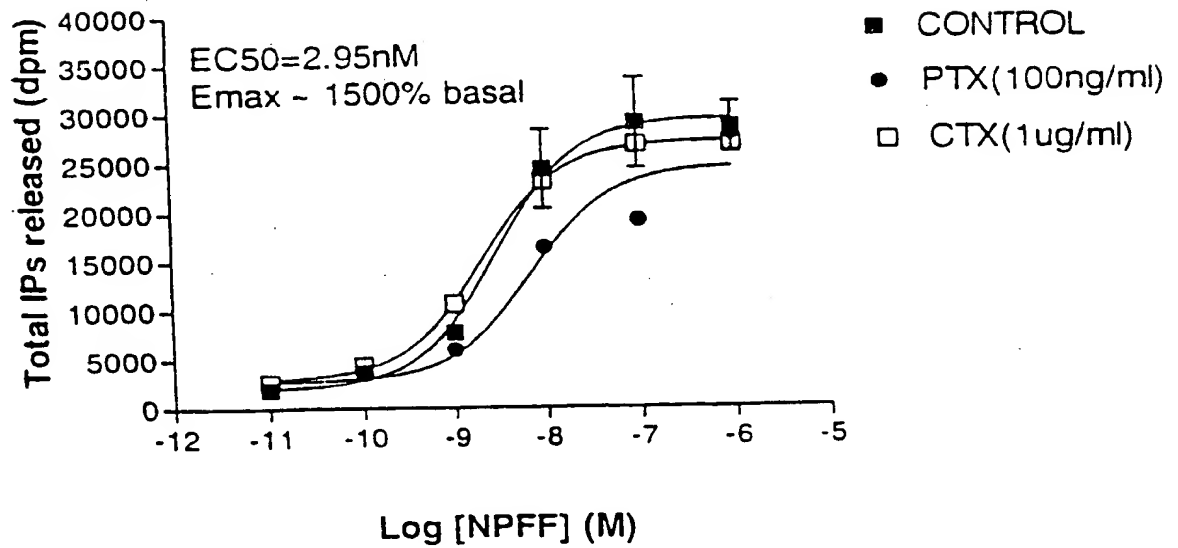
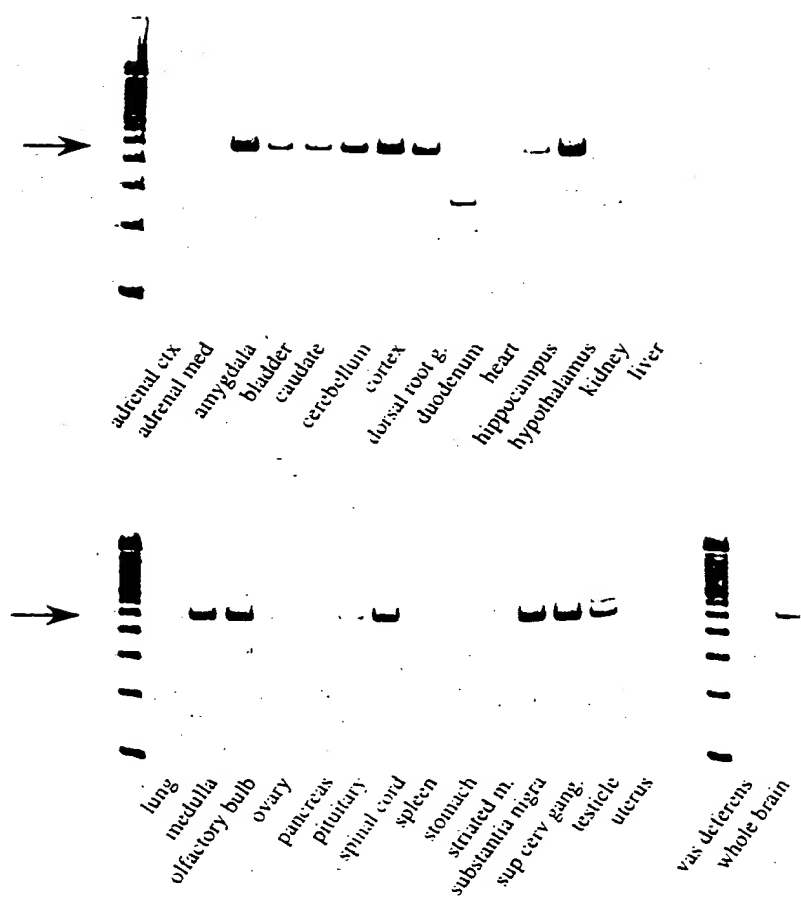


Figure 18B



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Figure 19



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Figure 20

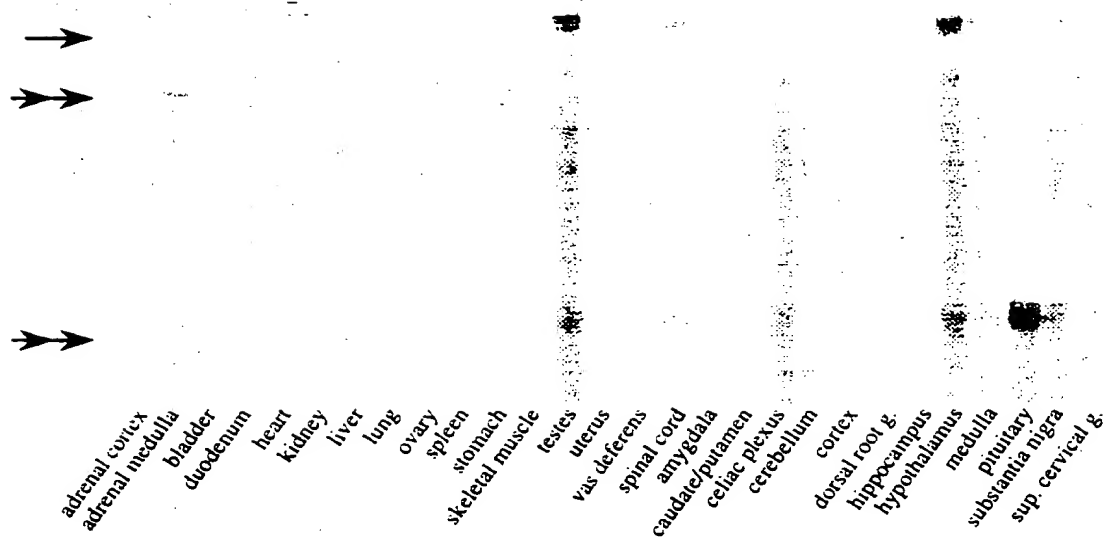




Figure 22A

1 AGCCTCTCCTTTGATAAGGTCCACCATGGGCAAGAGATGGGACTCAAACCTCTTCAGGAAG 60  
61 CTGGGATCACATCTGGAGTGGCAATGACACACAGCATCCTTGGTATTTCAGATATCAACAT 120  
121 CACATACATGAACACTACTATCTCCACCAGCCCCACGTGACAGCTGTCTTCATTAGCTCCTA 180  
181 CTTCCTGATCTTCTTCCCTGTGCATGGTGGGAAACACTGTCCGTTTGCTTTGTGTAATAAG 240  
241 GAATAGGTACATGCACACGGTCACATAATTCTTTCATCTTCAACCTCGCAATAAGTGACTT 300  
301 ACTGGTTGGAAATATTCTGCATGCCTATCACATTGCTGGACAACATCATAGCAGGATGGCC 360  
361 GTTTGGAAGCAGCATGTGCAAGATCAGCCGGCTGGTGCAAGGATATCGGTTGCCGCTTC 420  
421 TGTCTTACCTTGGTTGCCATAGCCGCTAGACAGATTCCGGTGTGTGGTCTACCCCTTTAA 480  
481 GCCCAAGCTCACTGTCAAGACAGCCCTTTGTCAATGATCGTGATCATCTGGGGCCTGGCCAT 540  
541 CACCATATGACCCCCATCTGCAATCATGTTACATGTACAGGAAGAAAAATACTACCGTGT 600  
601 GAGGCTCAGCTCCCAATAAAACCAGCACAGTCTACTGGTGTCTGGGAGGATTGGCCAAA 660

Figure 22B

661	CCAGGAAATGAGGAGGATCTACACCGTGCTCTTTGCCACTATCTACCTGGCTCCA	720
721	CTCCCTCATTTGTTATCATGTATGCAAGGATGGGGCTTCCCTCTTCAAGACCTCAGCACA	780
781	CAGCACAGGTAAGCAGCGCCCTGGAGCAGTGGCATGTATCCAAGAAACAGAAAGGTCAT	840
841	CAAGATGCTGCTGACTGTGGCCCTCCTTTTCATCCTTTCCCTGGCTTCCCCCTGTGGACTCT	900
901	GATGATGCTCTCAGACTATGCTGACCTGTCACTAACAACTACGTGTCATCAATATTA	960
961	TGTCACCCCTTTTGCCCCACTGGCTCGCCCTTCTGCAATAGCAGTGTCACCCCATCATTTA	1020
1021	TGGTTTCTTTAATGAAAAATTTTCGCAGTGGTTTCCAAGATGCTTTCCAGTTCTTGCCAAA	1080
1081	GAAAGTCAAACCCAGGAAGCCTATGGCCCTAAGAGCTAAACGCAACCTGGACATAAACAC	1140
1141	ATCTGGCCTGTTGGTCCATGAACCTGCATCTCAAAACCCCAAGTGGGGAAAACCTTGGGATG	1200
1201	TAGAAAAAGTGCAGACAAATCCCACACAGGAATCCTTGATGGAGGAACGGGAGAGCTAC	1260
1261	CAACAGTACTGAGACTTAGAAAGATAGTATGCTATCCCAATGTTATATAGCATACGAAGCC	1320

1334

Figure 22C

1321 AACTCCGATGGCTG

Figure 23A

1 M G K R W D S N S S G S W D H I W S G N 20  
21 D T Q H P W Y S D I N I T Y M N Y Y L H 40  
41 Q P H V T A V F I S S Y F L I F F L C M 60  
61 V G N T V V C F V V I R N R Y M H T V T 80  
81 N F F I F N L A I S D L L V G I F C M P 100  
101 I T L L D N I I A G W P F G S S M C K I 120  
121 S G L V O G I S V A A S V F T L V A I A 140  
141 V D R F R C V V Y P F K P K L T V K T A 160  
161 F V M I V I I W G L A I T I M T P S A I 180  
181 M L H V Q E E K Y Y R V R L S S H N K T 200  
201 S T V Y W C R E D W P N Q E M R R I Y T 220  
221 T V L F A T I Y L A P L S L I V I M Y A 240  
241 R I G A S L F K T S A H S T G K Q R L E 260  
261 Q W H V S K K K Q K V I K M L L T V A L 280  
281 L F I L S W L P L W T L M M L S D Y A D 300



Figure 23B

301	L	S	P	N	K	L	R	V	I	N	I	Y	V	Y	P	F	A	H	W	L	320
321	A	F	C	N	S	S	V	N	P	I	I	Y	G	F	F	N	E	N	F	R	340
341	S	G	F	Q	D	A	F	Q	F	C	Q	K	K	V	K	P	Q	E	A	Y	360
361	G	L	R	A	K	R	N	L	D	I	N	T	S	G	L	L	V	H	E	P	380
381	A	S	Q	N	P	S	G	E	N	L	G	C	R	K	S	A	D	N	P	T	400
401	Q	E	S	L	M	E	E	T	G	E	A	T	N	S	T	E	T				417

Figure 24A

hNPFF2 1 MNEKWDTNSSSENWHPiWNVNDTKHHLYSdINITVvNYyLHQpQvAAIFII 50  
|.:|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.:|  
rNPFF2 1 MGKRWDSNSSGSDHIWSGNDTQHPWYSdINITYmNYyLHQpHVtAVFIS 50

hNPFF2 51 SYFLIFFLCMMGNTVVCfIVMRNKHMHtVtNfILnLAISdLLvGIFcMP 100  
|.|.|.|.|.|.|.|.|.|.|.:|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|  
rNPFF2 51 SYFLIFFLCMVGNTVVCfVIRNRYMHtVtNfFIFnLAISdLLvGIFcMP 100

hNPFF2 101 ITLLDNIiAGWPFGNTMCKISGLVQGIsvAASvFTLVaiAvDRfQCvVYP 150  
|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|  
rNPFF2 101 ITLLDNIiAGWPFGSSMCKISGLVQGIsvAASvFTLVaiAvDRfRCvVYP 150

hNPFF2 151 FKPKLTiKtAFvIiMIiWVLaiTImSPsAvMLHVQeEKYyRVrLNSQnKT 200  
|.|.|.|.:|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|  
rNPFF2 151 FKPKLTvKtAFvMIvIiWGLaiTImTPsAiMLHVQeEKYyRVrLSShNKT 200

hNPFF2 201 SPVYwCREDwPNQeMRKiYtTVLFaniYLaPLSLiViMGRIGISLFRAA 250  
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rNPFF2 201 STVYwCREDwPNQeMRRIYtTVLFaTiYLaPLSLiViMAYARIGASLFKTS 250

**hnPFF2**   **251** VPHTGRKNQEQWHVVSRRKKQKI~~I~~KMLLIVALLFILSWPLWTLTMLSDYA   **300**

|| : . ||| | | : | | : | | | | | | | | | | | | | | | | | | | | | | | | | | | |

**rNPFF2**   **251** AHSTGKQRLEQWH.VSKKKQKVIKMMLTVALLFILSWPLWTLTMLSDYA   **299**

hNPFF2 301 DLSPNELQIINIYYPFAHWLAFGNSSVNP I I YGFFNENFRRGFQEAFQL 350  
|||||.|.:|||:||| ||| ||| ||| ||| ||| ||| ||| :||  
rNPFF2 300 DLSPNKLRVINIYVPFAHWLAFGNSSVNP I I YGFFNENFRSGFQDAF.. 347

hNPFF2 351 QLCQKRAKPMEAYALKA KASHVLINTSNQLVQESTFQNPHGETLLYRKSAE 400  
| | | : | | | : | | . | | | | | | | | | |  
rNPFF2 348 QFCQKKVKPQEAYGLRAKRNL DINTSGLLVHEPASQNP SGENLGCRKSAD 397

hNPFF2 401 KPQQLVMEELKETNSSEI  
| | | . | | | | | | |  
rNPFF2 398 NPTQESLMEETGEATNSTET

Figure 25A

rNPFF1 1 MEAEPSQPPNGSWPLQNGSDVETSMAT..SLTFSSYYQHSSPVAAMFIA 48  
| .||| .|.|. : . :||| | .|||. .  
rNPFF2 1 MGKRWDSNSSGSDHIWSGNDTQHPWYS DINITYMNYLHQPHVTA VFIS 50

rNPFF1 49 AYVLIFLLCMVGNTLVCFIVLKNRHMRTVTNMFILNLA VSDLLVGIFCMP 98  
.| ||| |||||. |||:|:|:| |||:|:|:| |||:|:|:| |||:|:|:|  
rNPFF2 51 SYFLIFFLCMVGNTVVCFVIRNRYMHTVTNFFIFNL AISDLLVGIFCMP 100

rNPFF1 99 TTLVDNLI TGWPF DNATCKMSGLVQGM SVSASVFTLV AIAVERFCIVHP 148  
||. ||:| |||| .. ||. |||||. ||. |||||. |||||. |||||. ||:|  
rNPFF2 101 ITLLDNIIAGWPFGSSMCKISGLVQGISVAASVFTLV AIAVDRFCVVYP 150

rNPFF1 149 FREKLT LRKALFTIAVIWALALLIMCPSAVT LTVT REEH.FMLDARNRS 197  
|: |||. :| :||| :|||: |||:| | |:|:| |. :|:|  
rNPFF2 151 FKPKLT VKTAFVMIVIIWGLAITIMTPSAIMLHVQEEKY YRVRLSSH NKT 200

rNPFF1 198 YPLYSCWEAWPEKGM RKVYTAVLF AHIYLVPLALIVMVYVRIARKLCQAP 247  
.| ||| |. ||:| |||| |||| |||||. ||||:| ||| |  
rNPFF2 201 STVYWCREDWPNQEMRR IYTTVL FATIYLA PLSLIVIMYARIGASLFKT. 249

Figure 25B

rNPFF1 248 GPARDTEEAEGGRTSRRRARVVHMLVMVALFFTLWLWVLLLLIDY 297  
| | . | : : : | : : | | | | | | | : : | | |  
rNPFF2 250 .SAHSTGKQRLQEQWHVSKKKQKVIKMLLTVALLFILSWLPLWTLMMMLSDY 298  
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rNPFF1 298 GELSEQLHLLSVYAFPLAHWLAFFHSSANPIIYGYFNENFRRGFQAAFR 347  
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rNPFF2 299 ADLSPNKLRVINIYVYFPAHWLAFCNSSVNPPIIYGFFNENFRSGFQDAF. 347  
| | . | : : : | : | | | | | . | | | | | : | | | | |  
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rNPFF1 394 GVPGPGRPLPLNRGRVAHQDGPGEGPCNHPMLTIPAWN 432  
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## SEQUENCE LISTING

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 35 40 45

Ala Tyr Val Leu Ile Phe Leu Leu Cys Met Val Gly Asn Thr Leu Val  
 50 55 60

Cys Phe Ile Val Leu Lys Asn Arg His Met Arg Thr Val Thr Asn Met  
 65 70 75 80

Phe Ile Leu Asn Leu Ala Val Ser Asp Leu Leu Val Gly Ile Phe Cys  
 85 90 95

Met Pro Thr Thr Leu Val Asp Asn Leu Ile Thr Gly Trp Pro Phe Asp  
 100 105 110

Asn Ala Thr Cys Lys Met Ser Gly Leu Val Gln Gly Met Ser Val Ser  
 115 120 125

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Ile Val His Pro Phe Arg Glu Lys Leu Thr Leu Arg Lys Ala Leu Phe  
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Glu Lys Gly Met Arg Lys Val Tyr Thr Ala Val Leu Phe Ala His Ile  
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 Arg Lys Leu Cys Gln Ala Pro Gly Pro Ala Arg Asp Thr Glu Glu Ala  
 245 250 255  
 Val Ala Glu Gly Gly Arg Thr Ser Arg Arg Arg Ala Arg Val Val His  
 260 265 270  
 Met Leu Val Met Val Ala Leu Phe Phe Thr Leu Ser Trp Leu Pro Leu  
 275 280 285  
 Trp Val Leu Leu Leu Leu Ile Asp Tyr Gly Glu Leu Ser Glu Leu Gln  
 290 295 300  
 Leu His Leu Leu Ser Val Tyr Ala Phe Pro Leu Ala His Trp Leu Ala  
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 Leu Pro Ser Glu Ser Gly Pro Ser Ser Gly Val Pro Gly Pro Gly Arg  
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&lt;400&gt; 3



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&lt;211&gt; 1302

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 5

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&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 6

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                     35                    40                    45  
 Ile Ile Ser Tyr Phe Leu Ile Phe Phe Leu Cys Met Met Gly Asn Thr  
                     50                    55                    60  
 Val Val Cys Phe Ile Val Met Arg Asn Lys His Met His Thr Val Thr  
                     65                    70                    75                    80  
 Asn Leu Phe Ile Leu Asn Leu Ala Ile Ser Asp Leu Leu Val Gly Ile  
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                     100                    105                    110  
 Phe Gly Asn Thr Met Cys Lys Ile Ser Gly Leu Val Gln Gly Ile Ser  
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 Val Ala Ala Ser Val Phe Thr Leu Val Ala Ile Ala Val Asp Arg Phe  
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                     145                    150                    155                    160  
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                     165                    170                    175  
 Pro Ser Ala Val Met Leu His Val Gln Glu Glu Lys Tyr Tyr Arg Val  
                     180                    185                    190  
 Arg Leu Asn Ser Gln Asn Lys Thr Ser Pro Val Tyr Trp Cys Arg Glu  
                     195                    200                    205  
 Asp Trp Pro Asn Gln Glu Met Arg Lys Ile Tyr Thr Thr Val Leu Phe  
                     210                    215                    220  
 Ala Asn Ile Tyr Leu Ala Pro Leu Ser Leu Ile Val Ile Met Tyr Gly  
                     225                    230                    235                    240  
 Arg Ile Gly Ile Ser Leu Phe Arg Ala Ala Val Pro His Thr Gly Arg  
                     245                    250                    255  
 Lys Asn Gln Glu Gln Trp His Val Val Ser Arg Lys Lys Gln Lys Ile  
                     260                    265                    270  
 Ile Lys Met Leu Leu Ile Val Ala Leu Leu Phe Ile Leu Ser Trp Leu

275

280

285

Pro Leu Trp Thr Leu Met Met Leu Ser Asp Tyr Ala Asp Leu Ser Pro  
 290 295 300

Asn Glu Leu Gln Ile Ile Asn Ile Tyr Ile Tyr Pro Phe Ala His Trp  
 305 310 315 320

Leu Ala Phe Gly Asn Ser Ser Val Asn Pro Ile Ile Tyr Gly Phe Phe  
 325 330 335

Asn Glu Asn Phe Arg Arg Gly Phe Gln Glu Ala Phe Gln Leu Gln Leu  
 340 345 350

Cys Gln Lys Arg Ala Lys Pro Met Glu Ala Tyr Ala Leu Lys Ala Lys  
 355 360 365

Ser His Val Leu Ile Asn Thr Ser Asn Gln Leu Val Gln Glu Ser Thr  
 370 375 380

Phe Gln Asn Pro His Gly Glu Thr Leu Leu Tyr Arg Lys Ser Ala Glu  
 385 390 395 400

Lys Pro Gln Gln Glu Leu Val Met Glu Glu Leu Lys Glu Thr Thr Asn  
 405 410 415

Ser Ser Glu Ile  
 420

&lt;210&gt; 7

&lt;211&gt; 1293

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 7

atggaggggg agccctccca gcctcccaac agcagttggc ccctaagtca gaatgggact 60  
 aacactgagg ccaccccggc tacaaacctc accttctcct cctactatca gcacacctcc 120  
 cctgtggcgg ccatgttcat tgtggcctat gcgctcatct tctgtctctg catggtgggc 180  
 aacaccctgg tctgtttcat cgtgctcaag aaccggcaca tgcatactgt caccaacatg 240  
 ttcatactca acctggctgt cagtgcctg ctggtgggca tcttctgcat gccaccacc 300  
 cttgtggaca acctcatcac tgggtggccc ttcgacaatg ccacatgcaa gatgagcggc 360  
 ttggtgcagg gcatgtctgt gtcggcttcc gttttcacac tgggtggccat tgctgtggaa 420  
 aggttccgct gcatcgtgca ccctttccgc gagaagctga ccctgcggaa ggcgctcgtc 480  
 accatcgccg tcactctggc cctggcgctg ctcatcatgt gtccctcggc cgtcacgctg 540  
 accgtcaccc gtgaggagca ccacttcatg gtggacgcc gcaaccgctc ctaccctctc 600  
 tactcctgct gggaggcctg gcccgagaag ggcacgcgca gggctctacac cactgtgctc 660  
 ttctcgcaca tctacctggc gccgctggcg ctcatcgtgg tcatgtacgc ccgcatcgcg 720  
 cgcaagctct gccaggcccc gggccccggc cccggggggc aggaggctgc ggacccgcga 780  
 gcatcgcggc gcagagcgcg cgtggtgcac atgctggtca tgggtggcgt gttcttcacg 840

```

ctgtcctggc tgcgctctg ggcgctgctg ctgctcatcg actacgggca gctcagcgcg 900
ccgcagctgc acctggtcac cgtctacgcc ttccccttcg cgcactggct ggccttcttc 960
aacagcagcg ccaaccccat catctacggc tacttcaacg agaacttccg ccgcggcttc 1020
caggccgcct tccgcgcccg cctctgcccg cgcccgctcg ggagccacaa ggaggcctac 1080
tccgagcggc ccggcgggct tctgcacagg cgggtcttcg tggtggtgcg gccacgcgac 1140
tccgggctgc cctctgagtc gggccctagc agtggggccc ccaggcccgg ccgcctcccg 1200
ctgcggaatg ggcgggtggc tcaccacggc ttgcccaggg aagggcctgg ctgctcccac 1260
ctgcccctca ccattccagc ctgggatata tga 1293

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&lt;210&gt; 8

&lt;211&gt; 430

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 8

```

Met Glu Gly Glu Pro Ser Gln Pro Pro Asn Ser Ser Trp Pro Leu Ser
  1                      5                      10                      15

```

```

Gln Asn Gly Thr Asn Thr Glu Ala Thr Pro Ala Thr Asn Leu Thr Phe
      20                      25                      30

```

```

Ser Ser Tyr Tyr Gln His Thr Ser Pro Val Ala Ala Met Phe Ile Val
      35                      40                      45

```

```

Ala Tyr Ala Leu Ile Phe Leu Leu Cys Met Val Gly Asn Thr Leu Val
      50                      55                      60

```

```

Cys Phe Ile Val Leu Lys Asn Arg His Met His Thr Val Thr Asn Met
      65                      70                      75                      80

```

```

Phe Ile Leu Asn Leu Ala Val Ser Asp Leu Leu Val Gly Ile Phe Cys
      85                      90                      95

```

```

Met Pro Thr Thr Leu Val Asp Asn Leu Ile Thr Gly Trp Pro Phe Asp
      100                      105                      110

```

```

Asn Ala Thr Cys Lys Met Ser Gly Leu Val Gln Gly Met Ser Val Ser
      115                      120                      125

```

```

Ala Ser Val Phe Thr Leu Val Ala Ile Ala Val Glu Arg Phe Arg Cys
      130                      135                      140

```

```

Ile Val His Pro Phe Arg Glu Lys Leu Thr Leu Arg Lys Ala Leu Val
      145                      150                      155                      160

```

```

Thr Ile Ala Val Ile Trp Ala Leu Ala Leu Leu Ile Met Cys Pro Ser
      165                      170                      175

```

```

Ala Val Thr Leu Thr Val Thr Arg Glu Glu His His Phe Met Val Asp
      180                      185                      190

```

Ala Arg Asn Arg Ser Tyr Pro Leu Tyr Ser Cys Trp Glu Ala Trp Pro  
 195 200 205  
 Glu Lys Gly Met Arg Arg Val Tyr Thr Thr Val Leu Phe Ser His Ile  
 210 215 220  
 Tyr Leu Ala Pro Leu Ala Leu Ile Val Val Met Tyr Ala Arg Ile Ala  
 225 230 235 240  
 Arg Lys Leu Cys Gln Ala Pro Gly Pro Ala Pro Gly Gly Glu Glu Ala  
 245 250 255  
 Ala Asp Pro Arg Ala Ser Arg Arg Arg Ala Arg Val Val His Met Leu  
 260 265 270  
 Val Met Val Ala Leu Phe Phe Thr Leu Ser Trp Leu Pro Leu Trp Ala  
 275 280 285  
 Leu Leu Leu Leu Ile Asp Tyr Gly Gln Leu Ser Ala Pro Gln Leu His  
 290 295 300  
 Leu Val Thr Val Tyr Ala Phe Pro Phe Ala His Trp Leu Ala Phe Phe  
 305 310 315 320  
 Asn Ser Ser Ala Asn Pro Ile Ile Tyr Gly Tyr Phe Asn Glu Asn Phe  
 325 330 335  
 Arg Arg Gly Phe Gln Ala Ala Phe Arg Ala Arg Leu Cys Pro Arg Pro  
 340 345 350  
 Ser Gly Ser His Lys Glu Ala Tyr Ser Glu Arg Pro Gly Gly Leu Leu  
 355 360 365  
 His Arg Arg Val Phe Val Val Val Arg Pro Ser Asp Ser Gly Leu Pro  
 370 375 380  
 Ser Glu Ser Gly Pro Ser Ser Gly Ala Pro Arg Pro Gly Arg Leu Pro  
 385 390 395 400  
 Leu Arg Asn Gly Arg Val Ala His His Gly Leu Pro Arg Glu Gly Pro  
 405 410 415  
 Gly Cys Ser His Leu Pro Leu Thr Ile Pro Ala Trp Asp Ile  
 420 425 430

&lt;210&gt; 9

&lt;211&gt; 23

&lt;212&gt; DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 9

gyntwryynn tnwsntgght ncc

23

<210> 10

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 10

avnadngbrw avannanngg rtt

23

<210> 11

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 11

ttatgcttcc ggctcgatg ttgtg

25

<210> 12

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 12

atgtgctgca aggcgattaa gttggg

26

<210> 13

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 13

ggtgctgctg ctgctcatcg actatg

26

<210> 14

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 14

ttggcgctgc tgtggaagaa ggccag

26

<210> 15

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 15

cggtgctctt cgcgcacatc tacc

24

<210> 16

<211> 60

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 16

tgccaagggg aaggcgtaga ccgacagcag gtgcagttgc agctcgatca gctccccata 60

<210> 17

<211> 53

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 17

ccacccttgt ggacaacctc atcactgggt ggcccttcga caatgccaca tgc

53

<210> 18

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 18

ctgctctgca tgggtgggcaa cacc

24

<210> 19

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 19

gacggcgatg gtagcagagcg c

21

<210> 20

<211> 65

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 20

gtcaccaaca tgttcatacct caacctggct gtcagtgacc tgctgggtggg catctttctgc 60  
atgcc 65

<210> 21

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 21

gcgagaagct gaccctgcgg aagg

24

<210> 22

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 22



tcgtcaccat cgccgtcatc tggg

24

<210> 23

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 23

cgatcatctgg gccgagggac acag

24

<210> 24

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 24

tgacggcgat ggtgacgagc gcc

23

<210> 25

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 25

cagcctccca acagcagttg gcc

23

<210> 26

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 26

tagcaaggat ccgcatatgg agggggagcc ctccc

35

<210> 27

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 27

cttcatgaat tcatcgcttg catgtatctc gtgtcc

36

<210> 28

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 28

cgtgtacggt gggaggtcta tataagcaga g

31

<210> 29

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 29

ccatcctaata acgactcact atagggc

27

<210> 30

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 30

actcactata gggctcgagc ggc

23

<210> 31

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 31

tgatagtgag ctttggttta aaagg

26

<210> 32  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 32  
gaagatctac accactgtgc tgtttg

26

<210> 33  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 33  
aacatctacc tggctcccct ctccc

25

<210> 34  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 34  
ttgtcatcat gtatggaagg attgg

25

<210> 35  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 35  
gaccacacac tggaacctat ctac

24

<210> 36  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 36

gcaattgcaa ctaacgtaaa gactg

25

<210> 37

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 37

tagcaaggat ccgaggttca tcatgaatga gaaatgg

37

<210> 38

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 38

cttcatgaat tcgcgtagta gagttaggat taccac

36

<210> 39

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 39

ctcctactac caacactcct ctcc

24

<210> 40

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 40

acgggttacg agcatccag

19

<210> 41  
 <211> 27  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: primer/probe

<400> 41  
 gatcagtgga ttggtccagg gaatatc

27

<210> 42  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: primer/probe

<400> 42  
 ccaggtagat gttggcaaac agcac

25

<210> 43  
 <211> 1334  
 <212> DNA  
 <213> Rattus norvegicus

<400> 43  
 agcctctcct ttgataaggt ccaccatggg caagagatgg gactcaaact cttcaggaag 60  
 ctgggatcac atctggagtg gcaatgacac acagcatcct tggatttcag atatcaacat 120  
 cacatacatg aactactatc tccaccagcc ccacgtgaca gctgtcttca ttagctccta 180  
 ctccctgacg ttcttcctgt gcatggtggg aaacactgtc gtttgctttg ttgtaataag 240  
 gaataggtac atgcacacgg tcaactaattt cttcatcttc aacctcgcaa taagtgactt 300  
 actggttgga atattctgca tgcctatcac attgctggac aacatcatag caggatggcc 360  
 gtttggaagc agcatgtgca agatcagcgg gctgggtgcaa gggatatcgg ttgccgcttc 420  
 tgtcttcacc ttggttgcca tagccgtaga cagattccgg tgtgtggtct acccctttaa 480  
 gcccaagctc actgtcaaga cagcctttgt catgatcgtg atcatctggg gcctggccat 540  
 caccattatg accccatctg caatcatggt acatgtacag gaagaaaaat actaccgtgt 600  
 gaggctcagc tcccacaata aaaccagcac agtctactgg tgtcgggagg attggccaaa 660  
 ccaggaaatg aggaggatct acaccacgt gctctttgcc actatctacc tggctccact 720  
 ctccctcatt gttatcatgt atgcaaggat tggggcttcc ctcttcaaga cctcagcaca 780  
 cagcacaggt aagcagcgcc tggagcagtg gcatgtatcc aagaagaaac agaaggtcat 840  
 caagatgctg ctgactgtgg cctcctttt catcctttcc tggcttcccc tgtggactct 900  
 gatgatgctc tcagactatg ctgacctgtc acctaacaaa ctacgtgtca tcaatattta 960  
 tgtctaccct tttgcccact ggctcgctt ctgcaatagc agtgtcaacc ccatcattta 1020  
 tggtttcttt aatgaaaatt ttcgcagtggt tttccaagat gctttccagt tctgccccaa 1080  
 gaaagtcaaa ccccaggaag cctatggcct aagagctaaa cgcaacctgg acataaacac 1140  
 atctggcctg ttggtccatg aacctgcatc tcaaaaccca agtggggaaa acttgggatg 1200  
 tagaaaaagt gcagacaatc ccacacagga atccttgatg gaggaacggg gagaagctac 1260  
 caacagtact gagacttaga aagatagtat gctatccaat gttatatagc atacgaagcc 1320

aactccgatg gctg

1334

&lt;210&gt; 44

&lt;211&gt; 417

&lt;212&gt; PRT

&lt;213&gt; Rattus norvegicus

&lt;400&gt; 44

Met Gly Lys Arg Trp Asp Ser Asn Ser Ser Gly Ser Trp Asp His Ile  
 1 5 10 15

Trp Ser Gly Asn Asp Thr Gln His Pro Trp Tyr Ser Asp Ile Asn Ile  
 20 25 30

Thr Tyr Met Asn Tyr Tyr Leu His Gln Pro His Val Thr Ala Val Phe  
 35 40 45

Ile Ser Ser Tyr Phe Leu Ile Phe Phe Leu Cys Met Val Gly Asn Thr  
 50 55 60

Val Val Cys Phe Val Val Ile Arg Asn Arg Tyr Met His Thr Val Thr  
 65 70 75 80

Asn Phe Phe Ile Phe Asn Leu Ala Ile Ser Asp Leu Leu Val Gly Ile  
 85 90 95

Phe Cys Met Pro Ile Thr Leu Leu Asp Asn Ile Ile Ala Gly Trp Pro  
 100 105 110

Phe Gly Ser Ser Met Cys Lys Ile Ser Gly Leu Val Gln Gly Ile Ser  
 115 120 125

Val Ala Ala Ser Val Phe Thr Leu Val Ala Ile Ala Val Asp Arg Phe  
 130 135 140

Arg Cys Val Val Tyr Pro Phe Lys Pro Lys Leu Thr Val Lys Thr Ala  
 145 150 155 160

Phe Val Met Ile Val Ile Ile Trp Gly Leu Ala Ile Thr Ile Met Thr  
 165 170 175

Pro Ser Ala Ile Met Leu His Val Gln Glu Glu Lys Tyr Tyr Arg Val  
 180 185 190

Arg Leu Ser Ser His Asn Lys Thr Ser Thr Val Tyr Trp Cys Arg Glu  
 195 200 205

Asp Trp Pro Asn Gln Glu Met Arg Arg Ile Tyr Thr Thr Val Leu Phe  
 210 215 220

Ala Thr Ile Tyr Leu Ala Pro Leu Ser Leu Ile Val Ile Met Tyr Ala  
 225 230 235 240  
 Arg Ile Gly Ala Ser Leu Phe Lys Thr Ser Ala His Ser Thr Gly Lys  
 245 250 255  
 Gln Arg Leu Glu Gln Trp His Val Ser Lys Lys Lys Gln Lys Val Ile  
 260 265 270  
 Lys Met Leu Leu Thr Val Ala Leu Leu Phe Ile Leu Ser Trp Leu Pro  
 275 280 285  
 Leu Trp Thr Leu Met Met Leu Ser Asp Tyr Ala Asp Leu Ser Pro Asn  
 290 295 300  
 Lys Leu Arg Val Ile Asn Ile Tyr Val Tyr Pro Phe Ala His Trp Leu  
 305 310 315 320  
 Ala Phe Cys Asn Ser Ser Val Asn Pro Ile Ile Tyr Gly Phe Phe Asn  
 325 330 335  
 Glu Asn Phe Arg Ser Gly Phe Gln Asp Ala Phe Gln Phe Cys Gln Lys  
 340 345 350  
 Lys Val Lys Pro Gln Glu Ala Tyr Gly Leu Arg Ala Lys Arg Asn Leu  
 355 360 365  
 Asp Ile Asn Thr Ser Gly Leu Leu Val His Glu Pro Ala Ser Gln Asn  
 370 375 380  
 Pro Ser Gly Glu Asn Leu Gly Cys Arg Lys Ser Ala Asp Asn Pro Thr  
 385 390 395 400  
 Gln Glu Ser Leu Met Glu Glu Thr Gly Glu Ala Thr Asn Ser Thr Glu  
 405 410 415

Thr

<210> 45  
 <211> 8  
 <212> PRT  
 <213> Rattus norvegicus

<400> 45  
 Phe Leu Phe Gln Pro Gln Arg Phe  
 1 5

<210> 46  
<211> 18  
<212> PRT  
<213> Rattus norvegicus

<400> 46  
Ala Gly Glu Gly Leu Ser Ser Pro Phe Trp Ser Leu Ala Ala Pro Gln  
1 5 10 15

Arg Phe

<210> 47  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 47  
tttgtcatta ttatgatcat ctgg

24

<210> 48  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 48  
aataaaaagc agggccacaa tcag

24

<210> 49  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 49  
tcattatttc ctactttctg atc

23

<210> 50  
<211> 24  
<212> DNA  
<213> Artificial Sequence



<220>

<223> Description of Artificial Sequence: primer/probe

<400> 50

ctcatttcct ggtttgcca atcc

24

<210> 51

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 51

tcttcaagac ctcagcacac agc

23

<210> 52

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 52

gagctggaaa gcttcttgga aacc

24

<210> 53

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 53

ctgggtgctcg gaggattggc caaaccagga aatgaggagg atctacacc

49

<210> 54

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 54

gcagtgtaa ccccatcatt tatgg

25

<210> 55  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 55  
caaagcaaac gacagtgttt cccacc 26

<210> 56  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 56  
agtgaccgtg tgcattgtacc tattcc 26

<210> 57  
<211> 60  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 57  
ggtgggaaac actgtcgttt gctttgttgt aataaggaat aggtacatgc acacgggtcac 60

<210> 58  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 58  
gtcacggatc cagcctctcc ttgataagg tccacc 36

<210> 59  
<211> 45  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 59

gtcagccatc gagttggctt cgtatgctat ataacattgg atagc

45

<210> 60

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 60

ctgggtcaccg tctacgcctt

20

<210> 61

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 61

ccgcggcgga agttct

16

<210> 62

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 62

acagcagcgc caaccccatc at

22

<210> 63

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 63

cctgattgtg gccctgct

18

<210> 64  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 64  
catttgagaga aaggtcagcg tag

23

<210> 65  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 65  
ctcatggctg cccctgtgga ctcaat

26

<210> 66  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 66  
gctgtggaaa ggttccgct

19

<210> 67  
<211> 17  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer/probe

<400> 67  
cgccttccga agggcca

17

<210> 68  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 68

atcgtgcacc ctttccgcga gaa

23

<210> 69

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 69

gaggatctac accaccgtgc tatt

24

<210> 70

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 70

gaagcccaaa tccttgcata c

21

<210> 71

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer/probe

<400> 71

tctacctggc tccactctcc ctcattggt

29

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/22384

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.2, 69.1, 320.1, 325, 348; 514/44; 530/350; 536/24.31; 800/3, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, MEDLINE, WEST

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ALLARD et al. Mechanisms Underlying the Cardiovascular Responses to Peripheral Administration of NPFF in the Rat. J Pharmacol Exp Ther. July 1995 Vol. 274, No. 1, pages 577-583.	84-90, 100-101, 103, 105-108, 110-111, 113
X	DEVILLERS et al. Characterization of a Potent Agonist for NPFF Receptors: Binding Study on Rat Spinal Cord Membranes. Neuropharmacology. May 1994, Vol. 33, No. 5, pages 661-669, especially page 665, Table I.	116-120, 140, 191, 194
X	PAYZA et al. Modulation of Neuropeptide FF Receptors by Guanine Nucleotides and Cations in Membranes of Rat Brain and Spinal Cord. J. Neurochem. May 1993, Vol. 60, No. 5, pages 1894-1899, especially page 1897, Figure 3.	116-120, 140, 191, 194



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 DECEMBER 1999

Date of mailing of the international search report

04 FEB 2000

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/22384

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUPOUY, V. et al. Species Differences in the Localization of Neuropeptide FF Receptors in Rodent and Lagomorph Brain and Spinal Cord. Peptides 1996, Vol. 17, No. 3, pages 399-405, especially page 400 Table II	75-76, 84-87, 100
A	KNAPP, RJ. Molecular biology and pharmacology of cloned opioid receptors. FASEB J. April 1995 Vol. 9, No. 7, pages 516-525 especially page 519, Figure 2	1-64, 72-76, 91-97, 99, 102, 109, 112, 115, 121, 126-128, 132-134, 142-145, 150-160, 166-174, 192-193, 195-196

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/22384

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 12-26, 37-39, 41-61 and 88-96 (IN PART)  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
Please See Extra Sheet.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-64, 72-76, 84-97, 99-103, 105-113, 115-212, 126-129, 132-134, 140-145, 150-160, 166-174, 191-196
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)\*



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/22384

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (7):

A61K 43/04; C07H 21/04; C07K 1/00, 14/00, 17/00; C12N 5/02, 5/06, 15/00, 09, 63, 70, 74; C12P 21/06; C12Q 1/68; G01N 33/00

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

435/6, 7.2, 69.1, 320.1, 325, 348; 514/44; 530/350; 536/24.31; 800/3, 21

## BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

### 2. Where no meaningful search could be carried out, specifically:

Claims 12-26, 37-39, 41-61 and 88-96 are unsearchable to the extent that they require reference to the specified sequences from the sequence listing. Because Applicant furnished a machine-readable copy of the sequence listing that contained errors, no meaningful search of the sequences per se can be carried out by this Authority. However, the subject matter of the claims has been searched to the extent possible with reference to the balance of the description.

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT-Rule 13:1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-64, 72-76 and 140-141, drawn to NPFF receptor DNA, vectors, host cells, NPFF receptor polypeptide, process of preparing polypeptide and pharmaceutical compositions.

Group II, claims 84-97, 99-103, 105-113, 115-121, 126-129, 132-134, 142-145, 150-160, 166-174 and 191-196, drawn to methods of identifying compounds.

Group III, claim(s) 70, 98, 104, 114, 135-137, 146-147, 161-163, 175, 181-182, 184-187 and 189-190, drawn to agonists of NPFF receptor and pharmaceutical compositions.

Group IV, claims 70, 98, 104, 114, 130, 148-149, 161, 164-165, 176, 181 and 183-190, drawn to antagonists of NPFF receptor and pharmaceutical compositions.

Group V, claims 78-83, drawn to transgenic animals.

Group VI, claims 124-125, drawn to methods of in vivo testing with transgenic animals.

Group VII, claims 65-69, 71 and 77, drawn to antibodies to NPFF receptor.

Group VIII, claims 122 and 138-139, drawn to methods of detection of NPFF receptor by hybridization.

Group IX, claim 123, drawn to method of detection of NPFF receptor with antibody.

Group X, claims 137 and 177-178, drawn to methods of treatment with agonist.

Group XI, claims 131 and 179-180, drawn to methods of treatment with antagonists.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Pursuant to 37 C.F.R. § 1.475 (d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first recited product, an isolated polynucleotide encoding NPFF receptor, a purified NPFF receptor polypeptide, expression vectors, process of preparing the purified polypeptide and pharmaceutical compositions. Further pursuant to 37 C.F.R. § (d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT Rule 13.2 and that each of such products and methods accordingly defines a separate invention.

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